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Pravastatin alters propranolol pharmacodynamics, but not pharmacokinetics, in the inflammation of pre-adjuvant arthritis.

By

©

John David Clements

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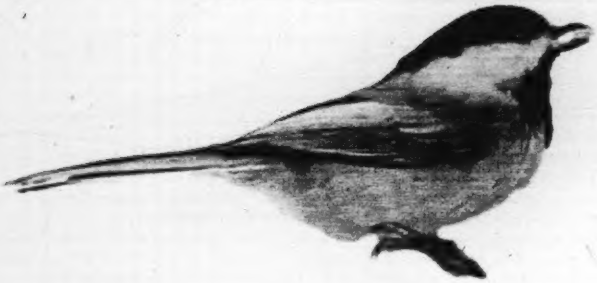

Dedications

To my dear Başo, for being my other half.

To my dear Mom and Dad for being such great parents and for making me who I am.

To Dr. Jamali for providing such great scientific inspiration.

Abstract

Rheumatoid arthritis increases cardiovascular morbidity and mortality by causing inflammation. Response to certain cardiovascular drugs, as measured by PR interval prolongation, is diminished in this population, and possibly contributes to excessive mortality. This occurs with verapamil, propranolol or sotalol in rats, and/or in humans, sometimes in spite of elevated blood concentrations of drug. Normalizing the cardiovascular response would be advantageous in rheumatoid arthritis patients as it may reduce cardiovascular risk, and therefore increase quality of life and life-span.

The statins have anti-inflammatory properties that have proven useful for the treatment of a wide range of diseases. In rheumatoid arthritis, these anti-inflammatory properties could potentially restore cardiovascular responsiveness. We used propranolol in the pre-adjuvant arthritis (Pre-AA) rat model of rheumatoid arthritis to test whether pravastatin restores diminished propranolol response, corrects altered inflammatory mediators, and normalizes propranolol pharmacokinetics. Sprague-Dawley rats were divided into four groups: Healthy/Placebo, Pre-AA/Placebo, Healthy/Statin, and Pre-AA/Statin. Despite a 10-fold increase in plasma propranolol concentrations, Pre-AA treated rats had significantly reduced propranolol responsiveness. Pravastatin normalized propranolol response but not its blood concentration. Proof of anti-inflammatory properties was demonstrated by the ability of pravastatin to reduce elevated serum interferon (IFN)-γ concentration in Pre-AA rats.

We have shown that pravastatin-treated inflamed rats were able to respond normally to propranolol despite the fact that IFN-γ concentrations remained much higher than in normal rats. From this, we concluded that more than one mechanism contributes to β_1-adrenergic receptor (AR) desensitization in inflammation.

Further investigation revealed that both norepinephrine transporter and β_1-AR densities were reduced in parallel in the cardiac tissue of inflamed animals. This observation suggested that activation of the sympathetic nervous system contributes to β_1-AR desensitization, and therefore propranolol response. Two observations support this idea. Sympathetic nervous system activation is a feature of rheumatoid arthritis. Second, our experiment with Pre-AA showed that inflammatory mediators were not strongly correlated with β_1-AR density, whereas norepinephrine transporter density was.

Our results suggest that pravastatin could be used for normalizing cardiac pharmacodynamic responsiveness in humans. The anti-inflammatory properties of pravastatin may achieve this end by directly lowering inflammation or by preventing the activation of the sympathetic nervous system. These findings could eventually be applied to clinical guidelines for the treatment of rheumatoid arthritis with the intent of improving cardiovascular risk reduction strategies.

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List of Abbreviations

AA	adjuvant arthritis
AUC	area under the curve
AUEC	area under the effect curve
AR	adrenergic receptor
$Ca_v1.2$	α_{1C} subunit of the L-type calcium channel
CRP	C-reactive protein
GRK	G-protein coupled receptor kinase
IFN	interferon
IL	interleukin
MMP	matrix metalloproteinase
NO_X^-	Nitric oxide metabolites (nitrite and nitrate)
PPAR	peroxisome proliferator-activated receptor
Pre-AA	pre-adjuvant arthritis
SEM	standard error of the mean
T_H	helper-inducer T lymphocyte
TNF	tumor necrosis factor

1. Introduction

Rheumatoid arthritis is an inflammatory disease that disfigures joints, causes pain, and shortens life-span, in part by increasing cardiovascular mortality. When compared to the healthy population, cardiovascular disease accounts for approximately 35-50% of the excessive mortality that exists in these patients.[1] Forty percent of rheumatoid arthritis patients take anti-hypertensive agents,[2] which highlights their elevated baseline risk for cardiac death. Inflammatory mediators that are common to cardiovascular disease and rheumatoid arthritis may contribute to elevated heart disease in rheumatoid arthritis patients. Moreover, inflammatory status is a main determinant of clinical outcome in patients with severe unstable angina.[3] Also, inflammation associated with rheumatoid arthritis may interfere with the successful treatment of cardiovascular disease.[4] There is evidence that rheumatoid arthritis patients are relatively resistant to cardiovascular drug pharmacotherapy, and therefore, the ability to restore the normal pharmacodynamic response to such drugs in these patients would be highly desirable.

How to resolve the issue of reduced pharmacodynamic response to cardiovascular drugs in rheumatoid arthritis patients is not known. One approach would be to reduce the severity of inflammation by administering anti-inflammatory agents. Preferable would be a drug that has a wide margin of safety. The hydroxymethylglutaryl co-enzyme A reductase inhibitors, i.e. the statins, are currently used to reduce cardiovascular disease risk by lowering cholesterol. The statins also possess many anti-inflammatory properties.[5] By reducing inflammatory mediators such as interleukin (IL)-6 and interferon (IFN)-gamma, the statins may also prove useful in restoring cardiovascular drug response in inflammatory diseases. This is my rationale for investigating whether the use of pravastatin normalizes the pharmacodynamic response to cardiovascular drugs in rheumatoid arthritis patients.

We are also interested in the mechanisms by which inflammation down-regulates cardiovascular responsiveness to certain cardiovascular drugs. Another part of my work will investigate the influence of statin administration on two aspects of cardiovascular health. The first aspect is how statins influence pro-inflammatory and anti-inflammatory mediators. The second aspect is how statins influence the profile of proteins involved in beta-1-adrenergic receptor signaling. Proteins of particular interest are the norepinephrine transporter, the L-type calcium channel, G-protein coupled receptor kinase, and the beta-1 adrenergic receptor protein.

1.1. *Inflammation*

Immune cells and inflammatory mediators induce *dolor, rubor, calor, and tumor* (referring to pain, redness, heat, and swelling, respectively) in response to tissue injury, pathologic conditions, or infection.[6, 7] Inflammation helps to repair damaged tissue by replacing and removing dead, un-viable, or damaged cells. A step-wise process is initiated, whereby vessel permeability increases; circulating 'inflammatory' cells attach to the vessel lining; inflammatory mediators are released; apoptosis (and preferably not necrosis) is initiated; finally leading to cell replacement and re-vascularization.[8]

1.1.1. Mechanisms of inflammatory response

The innate immune system, also known as the nonspecific immune system, is the first line of defense against pathogens. Cellular components, acute phase proteins, physical barriers, and even fever, are all considered integral to non-specific defense mechanisms. Immune cells of the nonspecific immune system specialize in phagocytosis, and examples include blood monocytes, tissue macrophages, and neutrophils.[7]

The adaptive immune system has four main attributes that separate it from the innate immune system: 1) diversity of challenges, 2) antigenic specificity, 3) immunologic memory, and 4) self versus nonself recognition. Three major groups of cells of adaptive immunity include antigen presenting cells, B lymphocytes (B cells), and T lymphocytes (T cells).[7] Antigen presenting cells 'present' processed protein as antigens to T cells which then help immune cells to differentiate and specialize. Antigen presenting cells are comprised of B cells, dendritic cells, and macrophages. Because macrophages are important to both innate and adaptive immunity, the macrophage serves as a bridge between the two immune systems. B cells elicit adaptive humoral responses by differentiating into antibody-secreting plasma cells. T cells induce cellular immunity by differentiating into cytotoxic T lymphocytes. Alternatively, T cells differentiate into helper-inducer T lymphocytes (T_H cells). T_H cells, whether class one (T_H1) or class two (T_H2), secrete cytokines (see Table 1-1 for a list of cytokine descriptions) which either enable or inhibit various immune cells.[7]

Table 1-1. Select immune cell-derived cytokines and their primary biologic activity.

Cytokine	Origin/ Classification	Primary Biologic Activity
IL-1	Activated macrophages	Stimulation of T- and B cells. Acute-phase response, including increased production of C-reactive protein.
IL-2	T_H1, T_H2	Proliferation of activated T cells and B cells.
IL-3	T cells	Hematopoietic growth factor.
IL-4	T_H2	**Hallmark T_H2 cytokine.** B cell proliferation.
IL-5	T_H2	Eosinophil growth and function.
IL-6	T_H2	B cell proliferation. Ig production. Acute-phase response.
IL-7	Thymic cells, bone marrow stromal cells	Growth factor for early B and T lymphocytes.
IL-8	Macrophages	Chemoattractant for T cells.
IL-10	T_H2	Inhibits cytokine production by T_H1 cells and macrophages. Promotes B cell proliferation and antibody responses. Downregulates cell-mediated immunity.
IL-12	T_H1	Secreted by T_H2 cells. Stimulates IFN-γ production. Enhances T_H1 response.
IL-13	T_H2	Stimulates B cell proliferation.
TNF-α	Activated macrophages	Tumor necrosis. Vascular thrombosis. Mediates host response to gram negative bacteria.
IFN-γ	T_H1	**Hallmark T_H1 cytokine.** Inhibits Th2 cells. Induction of cell-mediated immunity. Anti-viral. Anti-tumor. Activation of macrophages, neutrophils, and natural killer cells.
TGF-β	T_H1/T_H2	Inhibits cytokine release and lymphocyte proliferation.

Table adapted from references [7, 9-11].

The release of cytokines from various immune cells, and other cells, aids in the initiation, propagation, and regulation of immune response. T_H1 and T_H2 cells collectively help to initiate specific immune responses. T_H1 cells and macrophages that secrete IL-2, IL-12, and IFN-γ promote inflammation and cell-mediated responses. In contrast, T_H2 cells secrete IL-4, IL-5, IL-10, and IL-13 that help initiate the B cell response (humoral/antibody).[12-15] T_H2 cells reduce macrophage activity. Therefore, T_H1-mediated responses are best suited for protection from viruses, cancers, and intra-cellular pathogens. T_H2-mediated responses are best suited for countering extracellular pathogens.[9]

Depending on the cytokine profile that T_H precursor cells (naïve CD4+ T cells) are exposed to, differentiation will lead to either a T_H1 or T_H2 cell. This results in a skewing of the T_H cell population.[16] This is a tightly controlled process, and it depends on the type of antigen presenting cell interaction, the co-stimulatory molecules present, the strength of the T cell receptor stimuli, and the early stage cytokine exposure of the T_H precursor. These factors dictate whether the naïve CD4+ T cell will develop into a T_H1 or T_H2 cell.[15, 17] When the inflammatory insult or challenge has ended, it is of utmost importance that the system returns to its normal state. Continued, and inappropriate, skewing of the T_H cell population may contribute to chronic disease.

T_H1 and T_H2 responses are in balance with one another. As one T_H cell population response strengthens, the other T_H cell population weakens. For example, exogenously introduced IL-4 and IL-10 (both Th2 cytokines) effectively inhibits the development of a T_H1 response in murine collagen-induced arthritis or in human monocytes ex vivo.[14, 15, 18] Conversely, IFN-γ (the hallmark T_H1 cytokine) blocks the proliferation of T_H2 cells.[17] Interference with the regulatory processes governing T_H1/T_H2 balance can result in T_H-skewed diseases, such as rheumatoid arthritis or asthma.[14, 16, 19-21] Table 1-2 lists some examples of T_H1 and T_H2 diseases.

Table 1-2. Diseases or conditions associated with a T_H1/T_H2 imbalance.

	T_H1-Skewed Profile	T_H2-Skewed Profile
Disease or condition	Type 1 diabetes mellitus*	Transplantation *tolerance*
	Multiple sclerosis*	*Successful* pregnancy
	Crohn's disease	Allergy
	Rheumatoid arthritis*	Asthma
	Unexplained abortions	Systemic Lupus Erythematosus
	Atherosclerosis	

*may be mixed T_H1/T_H2, but is generally a T_H1-skewed profile. Table adapted from references [9, 22, 23].

Generalizing rheumatoid arthritis in terms of T_H1/T_H2 skewing is useful for simplicity sake. However, alternative mechanisms may influence the course of the disease independent of T_H1/T_H2 skewing. For example, glutathione redox status in oxidative stress influences whether macrophage populations become T_H1-skewed or T_H2-skewed. High concentrations of reduced glutathione skews immune cell towards a T_H1 profile, whereas high concentrations of oxidized glutathione skews immune cells towards a T_H2 profile.[9] Also, eicosapentaenoic acid and docosahexaenoic acid can improve inflammatory disease without altering the T_H1/T_H2 ratio.[9] Moreover, some diseases that are labeled as T_H1 in the literature, in fact have a slightly mixed T_H1 / T_H2 cytokine profile (Table 1-2).[9] Despite these exceptions, however, it is still helpful to classify rheumatoid arthritis as being T_H1-skewed.

In summary, there are two main consequences of T_H1/T_H2 immune cell imbalance. First, one immune cell type can suppress the other. Second, skewing of T_H cells influences the development and outcome of many diseases. While the focus of the next section is not always on the balance of T_H1 and T_H2 immune cells, it is important to keep this concept in mind.

1.1.2. Diseases of inflammatory origin

Normally inflammatory processes are self-limiting. At times, however, inflammation may progress to a chronic state. Examples of chronic diseases of inflammatory origin include rheumatoid arthritis, gout, Crohn's disease, diabetes, stroke, myocardial infarction, cancer, and hypertension: this list is not all-inclusive.[8] 'Inflammation' is therefore present, and central, in many pathophysiological conditions. In this thesis we will focus on inflammation associated with rheumatoid arthritis and cardiovascular disease.

The list below briefly describes inflammatory processes in a range of diseases. Please note the broad range of pathophysiological processes, the similarities amongst the inflammatory mediators.

- Atherosclerosis – IFN-γ and other inflammatory cytokines cause an increase the uptake of lipids in damaged arterial walls.[24]

- Hypertension – angiotensin II encourages the formation of reactive oxygen species and pro-inflammatory cytokines, such as IL-6. Nuclear factor-κB increases the expression of pro-inflammatory cytokines.[25] Activation of the transcription factor nuclear factor-κB is a major contributor to the pathophysiology of hypertension.[26]
- Diabetes – hyperglycemia promotes glycation of macromolecules and the subsequent activation of nuclear factor-κB.[27]
- Obesity – adipose tissue is a source of tumor necrosis factor-α (TNF-α) and IL-6, and therefore stimulates atherosclerosis.[28]
- Infection – chronic infections, including prostatitis, gingivitis, and bronchitis, enhance the production of circulating inflammatory cytokines. This supports atherosclerotic plaque development.[24]
- Rheumatoid arthritis – high concentrations of circulating IL-6 and C-reactive protein (CRP) may contribute to increased cardiovascular mortality.[29]
- Colon cancer – IL-6 signaling is important in the maintenance of colon cancer, just as it is with rheumatoid arthritis, inflammatory bowel disease, and asthma.[30]

1.1.2.1. Cardiovascular disease

Many cardiovascular diseases have an inflammatory component. For example, circulating cytokines are elevated in patients with acute myocardial infarction, angina, percutaneous coronary intervention, and heart failure.[31, 32] The importance of inflammatory mediators in cardiovascular disease is highlighted by the fact that they are often predictive of clinical outcome. Inflammatory processes involved in cardiovascular disease are described in this section. The focus of this section is on atherosclerosis because it is a precursor to many cardiovascular diseases.

Cardiovascular inflammation associated with atherosclerosis is characterized by increased cytokine production, endothelial dysfunction, hypercoagulability, and the recruitment of immune cells.[24, 33] The integrity of the endothelium is of utmost importance in cardiovascular health because it influences vascular tone, thrombosis development, and leukocyte trafficking to sites of injury.[34] Risk factors for atherosclerotic plaque development include elevated and modified low-density lipoprotein, cigarette smoking, hypertension, diabetes mellitus, elevated plasma homocysteine, and *Chlamydia pneumoniae* infection.[35] The endothelial dysfunction of early atherosclerosis is associated with changes in nitric oxide signaling and processing. Diminution of nitric oxide production, or its reduced bioavailability, may ultimately result in endothelial dysfunction.

Atherosclerotic lesions are infiltrated by leukocytes, even in early lesions.[36] This demonstrates the inflammatory nature of atherosclerosis. Leukocytes do not normally bind with the endothelial lining of healthy blood vessels. Unhealthy vessel walls, however, express high levels of vascular adhesion molecule-1,[24, 37] which attracts inflammatory cells and platelets to the area, and increases endothelial permeability.[24, 33, 38] Subsequent leukocyte migration across the vascular wall,[36] establishes a strong inflammatory basis for atherosclerosis.

Injury of vascular endothelium causes macrophages and certain T cells to release cytokines and growth factors. Uyemura et al have shown that atherosclerosis is a T_H1-skewed disease in which the T_H1/T_H2 balance influences immune-mediated tissue injury. Within atherosclerotic plaques T_H1 cytokine production (promoted by IL-12) predominates over T_H2 cytokine production.[18] T cells become one of the most common cell types in atherosclerotic plaques, so any alteration of the T cell profile can influence disease progression.[39, 40] Consistent with atherosclerosis being a T_H1 disease, treatment with IL-10, an anti-inflammatory T_H2 cytokine, initiates improvements in atherosclerosis.[32] Further, even the arteries of young children can become ‘inflamed’ and develop fatty streaks. Fatty streaks are of inflammatory origin, consisting largely of monocyte-derived macrophages and T lymphocytes.[35, 41]

The recruitment and proliferation of smooth muscle cells within atherosclerotic lesions signals the beginning of vascular remodeling. Cytokines, chemokines, and growth factors further damage the vessel wall. The ensuing necrosis and fibrosis destabilize the plaque, which is a situation conducive to thrombus formation. Lipid accumulation in atherosclerotic plaques is also important. Much of the lipid is taken up by macrophages that eventually morph into foam cells. A mature atherosclerotic plaque forms as the atheromatous core becomes necrotic and fills with cell debris, lipids, and cholesterol.[42]

The matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases. Enzymes of this group remodel the extracellular matrix in angiogenesis, heart disease, inflammation, wound healing, embryogenesis, and other processes.[43] MMPs are secreted in an inactive form by many cellular sources including endothelial cells, macrophages, and T cells.[44] The inactive, pro-enzyme, form of the MMPs can be activated by proteolytic cleavage or by reaction with peroxynitrite, which is an inflammatory mediator.[45] The activity of MMPs is balance by it natural inhibitor, tissue inhibitors of matrix metalloproteinases, ie. the TIMPs.[46]

MMPs play a major role in cardiovascular disease, such as atherosclerosis, myocardial infarction, and cardiac hypertrophy.[44] Plaque stability in atherosclerosis is important for preventing blood clot formation and thrombosis. Collagen helps provide strength and stability to atherosclerotic plaques, and its degradation by MMPs is deleterious. MMP activity is heightened in the vasculature in atherosclerosis, and contributes to plaque instability and plaque rupture.[44]

In addition to matrix targets, MMP-2 also has non-matrix extracellular targets that include IL-1β precursor, big endothelin-1, and monocyte chemoattractant protein-3.[43] Therefore, MMP-2 also exerts influence over inflammation and vascular tone in cardiac tissue and vessels. In addition to extracellular targets, MMP-2 also has intracellular targets. These targets include troponin I[47] and myosin light chain-1,[48] and therefore influence myocardial contractility, particularly in ischemia-reperfusion injury. The measurement of MMP-2, therefore, may provide insight into the mechanisms of altered pharmacodynamic response in inflammatory diseases since it has both intra- and extracellular effects.

Excessive mortality is associated with inflammation. The circulating inflammatory marker CRP is a strong, and cholesterol-independent, predictor of both myocardial infarction and stroke. This is true for both men and women, even in those who are apparently healthy.[49, 50] Inflammation contributes to cardiovascular risk by oxidizing low-density lipoproteins in the vessel wall. This contributes to monocyte recruitment, intima infiltration, secretion of pro-inflammatory cytokines, proliferation of smooth muscle cells, loss of endothelial function, and activation of the clotting system. Furthermore, inflammatory mediators can activate MMPs, thereby undermining the integrity of the plaque's fibrous cap, resulting in plaque fissure or rupture.

1.1.2.2. Rheumatoid arthritis

Originating in the New World, rheumatoid arthritis emerged in Europe in the early parts of the 19th century. Currently, rheumatoid arthritis has a prevalence of approximately 1%.[51] Rheumatoid arthritis causes pain and suffering in a great number of people, reducing not only quality of life, but also causing permanent disability in 31-42% of patients.[52] Rheumatoid arthritis is an inflammatory disease characterized by symmetrical synovitis, which upon progression destroys joints via cartilage and bone erosion.[53] High concentrations of cytokines cause pannus formation, tissue-destructive enzyme creation, and inappropriate vascularization. Additionally, excessive cytokine production and inflammation are thought to contribute to the extra-articular manifestations of rheumatoid arthritis.[54] Extra-articular manifestations of rheumatoid arthritis include, amongst others, pericardial inflammation, systemic vasculitis, rheumatoid nodule formation, splenomegaly, granulocytopenia, and Sjögren's syndrome.[54]

Rheumatoid arthritis significantly decreases quality of life by causing pain and physical impairment, which in turn reduces independence. Swollen and tender joints, extra-articular manifestations, and the crippling effect of the disease, contribute to reductions in World Health Organization Quality of Life scores in the following categories: Physical health, Psychology, Social relationships, and Environmental.[55] It is therefore not surprising that patients also have reductions in life-span approaching 3-10 years.[56]

1.1.2.2.1. Epidemiology and etiology of rheumatoid arthritis

Rheumatoid arthritis affects a large number of people throughout North America. Approximately 0.5-1.0% of this population have rheumatoid arthritis, with an annual incidence of 0.02-0.05%. These rates are in contrast to other parts of the world where rheumatoid arthritis is less common. For instance, in Southern Europe and in developing countries the prevalence is only 0.3-0.7%.[56] Fortunately, rheumatoid arthritis incidence and severity is falling with time. The incidence of rheumatoid arthritis in the women of Rochester, USA, has fallen from 83/100,000 in 1955-1964, to 40/100,000 in 1980-1994.[51]

There are a number of possible explanations for discrepancies in the prevalence of rheumatoid arthritis amongst earth's populations. These explanations may be routed in the etiology of the disease. Rheumatoid arthritis has a hereditary component. Therefore, ethnic genetic variations are important. The genetic component of rheumatoid arthritis is partly explained by population differences in the presence of 'rheumatoid epitopes' and in polymorphisms of TNF-α.[57] A single nucleotide polymorphism at the PTPN22 gene (a rheumatoid arthritis susceptibility gene) results in an alternate form of a protein tyrosine phosphatase.[57] This polymorphism is strongly associated with the susceptibility for rheumatoid arthritis development. The protein tyrosine phosphatase polymorphism is also strongly associated with other autoimmune diseases including juvenile idiopathic arthritis, systemic lupus erythematosus, Graves' disease, and Addison's disease.[57]

Differential rates of viral and bacterial infection, from population to population, around the globe may also contribute to varied rates of rheumatoid arthritis. For example, research has established associations between rheumatoid arthritis and parvovirus, rubella virus, Epstein-Barr virus, and *Borrelia* *burgdorferi*. However, this theory is being debated since there are not good temporal associations.[57]

Smoking is a modifiable risk factor for rheumatoid arthritis that has been shown, in longitudinal studies, to increase the risk of rheumatoid arthritis in a dose-dependent manner. Therefore, differences in smoking rates may also change rheumatoid arthritis rates.[57]

Females are at higher risk of developing rheumatoid arthritis, but it is not known whether female sex hormones are responsible. However, as with the elderly, women are more likely to have higher disease severity.[51, 56] Low socioeconomic status is also associated with poor prognosis, but it may not be associated with higher prevalence.[57]

Many factors contribute to excessive cardiovascular disease in rheumatoid arthritis patients.[23] Although atherosclerosis is one of the main causes of additional cardiovascular disease, other explanations exist. These explanations include high levels of the receptor for advanced glycation end products (also known as RAGE), blood homocysteine (associated with methotrexate use), blood lipids, pro-thrombotic factors, and markers of inflammation including erythrocyte sedimentation rate, CRP, IL-6, and serum amyloid A.[58-60] Rheumatoid arthritis patients also have excessive arterial thickness, endothelial dysfunction, and central blood pressure.[60, 61] Finally, glucocorticoid use causes a threefold increase in cardiovascular event rates (95% CI, 1.81-5.18) in rheumatoid factor positive patients, but not rheumatoid factor negative patients.[62] Even adjusting for known risk factors, women who have had rheumatoid arthritis for longer than 10 years have a 3.1 fold increase (95% CI, 1.64 to 5.87) in the incidence of myocardial infarction.[63] Furthermore, the incidence of sudden cardiac death is elevated in rheumatoid arthritis patients.[64] Importantly, sudden cardiac death is usually associated with arrhythmia.

1.1.2.2.2. Inflammatory profile of rheumatoid arthritis

T lymphocytes are one of the predominant immune cell types located in the inflamed synovium of rheumatoid arthritis patients. Consequently cytokines are released in large quantities from synovial tissue. Essentially, synovium is morphed into lymphoid tissue, and is therefore unable to serve its normal function of joint articulation.[65] Importantly, the release of pro-inflammatory T_H1 cytokines (IFN-γ, and IL-2) predominates over the release of anti-inflammatory T_H2 cytokines (IL-4 and IL-5). Excessive T_H1 skewing is associated with increasing disease severity.

B lymphocyte activity is also important in the progression of rheumatoid arthritis. In fact, the failure of immune tolerance by the B cell system is thought to be an early pathogenic step in rheumatoid arthritis development. The lack of B cell tolerance is detrimental because auto-antibodies can be produced. These antibodies then attack self-antigens, which, in turn, cause inflammatory damage. Therefore, the B cell is an integral component of joint inflammation in rheumatoid arthritis.[65]

The structure and function of articular joints are closely linked. Any degradation in structure is closely followed by decreases in function. The synovium of articular joints is normally populated with macrophage-like and fibroblast-like synoviocytes. During the inflammatory process of arthritis, a heterogeneous collection of immune cells infiltrate the synovium, thereby disturbing the structure of the synovium.

Rheumatoid arthritis is associated with structural changes in bone. Infiltration of bone by B cells, T cells, and macrophages,[65] causes excessive cytokine signaling and the subsequent activation of osteoclasts. Osteoclast activation in rheumatoid arthritis accelerates bone degradation.[66] Additionally, MMP activation contributes to the structural breakdown of connective tissue by enzymatically digesting proteins such as collagen.[65]

Three distinct types of bone loss occur in rheumatoid arthritis. Focal bone loss is associated with early rheumatoid arthritis and increased mortality. It is mediated by excessive osteoclast activity, especially at pannus-bone interfaces. Focal bone loss is associated with poor functionality because of subsequent joint deformation. Therapeutically, bisphosphonate administration is associated with significant reductions in focal bone erosion, even in the presence of inflammation. Second, periarticular bone loss in rheumatoid arthritis occurs in trabecular bone which is adjacent to the inflamed joint. Inflammation in this area heightens vascularization and decreases joint mobility. Finally, generalized bone loss occurs at sites distal to inflammatory foci. This is characterized by bone resorption rates that exceed bone deposition rates, and puts rheumatoid arthritis patient at heightened risk of vertebral and hip fractures.[66] Generalized bone loss may also be attributable to reduced mobility.

Inflammatory cells that reside in arthritic joints secrete vast amounts and varieties of inflammatory mediators. When the density of the receptor activator for nuclear factor-κB ligand (RANKL) is increased in inflamed joints, osteoclasts become activated. Infiltrating macrophages secrete TNF-α, thereby causing both indirect (via RANKL) and direct activation of osteoclasts. This explains why anti-TNF-α therapy with etanercept, adalimumab, and infliximab is able to reduce focal bone erosion in rheumatoid arthritis.[66] Other cytokines and mediators of inflammation involved in bone erosion include IL-1, IL-6, IL-17, and prostaglandins.[66] The administration of the IL-1 antagonist, anakinra, is proven safe and effective in the treatment of rheumatoid arthritis in humans because it reduces IL-1 secretion in joints and bones.[67]

1.1.2.2.3. Rheumatoid arthritis alters the cardiovascular system

In addition to joint pain, disfigurement, and disability, rheumatoid arthritis also increases cardiovascular-related mortality.[68, 69] The link between inflammation and cardiovascular disease first received attention in the 1980's,[70-72] and it has since been determined that rheumatoid arthritis patients have an estimated shorter life expectancy that ranges from 3 to 18 years (depending on the study), with 35 to 50% of the increased risk of death being attributable to cardiovascular disease.[59, 68, 69] In fact, compared to the general population, rheumatoid arthritis patients have a two-fold to four-fold higher myocardial infarction rate.[73]

The mechanism of increased mortality in rheumatoid arthritis is unknown. Possible explanations include diminished physical exercise, excessive atherosclerosis, arterial thickening, or possibly the independent association between cardiovascular risk and pro-inflammatory mediators, such as CRP,IL-6, IFN-γ, and TNF-α.[23, 59, 61, 68, 74, 75] These markers of inflammation are not unique to rheumatoid arthritis and are implicated in cardiovascular disease. Activation of the sympathetic nervous system in rheumatoid arthritis may also contribute to excessive cardiovascular death rates. Sympathetic nervous system activation is associated with an increased risk of arrhythmia-related sudden cardiac death.[76] This prevalence of atherosclerosis is heightened in rheumatoid arthritis patients in a manner independent of classic atherosclerosis risk factors, such as hyperlipidemia. As mentioned in Section 1.1.2, endothelial dysfunction in blood vessels is associated with poor nitric oxide signaling and the development of atherosclerosis. Vessels are not able to readily vasodilate, and as leukocytes and platelets adhere to the endothelium, the risk of cardiovascular events becomes exaggerated.

Congestive heart failure is highly prevalent in rheumatoid arthritis patients. Most congestive heart failure in rheumatoid arthritis patients is characterized by left ventricular dysfunction, in particular the relaxation and filling mode that occurs during diastole.[70] Diastolic dysfunction can occur at any point in rheumatoid arthritis progression, from early to late rheumatoid arthritis. Diastolic dysfunction can even precede symptomatic rheumatoid arthritis.[70]

The risk of developing congestive heart failure is highest in rheumatoid arthritis patients who have the highest degree of inflammation. Those having the highest concentrations of CRP, IL-6, and TNF-α, compared to those with the lowest concentrations, have a 2-4 fold increase in the risk of developing congestive heart failure.[73] One might anticipate that anti-TNF-α therapy is beneficial in the treatment of congestive heart failure. However, anti-TNF-α therapy in congestive heart failure has not proven useful, even though it is beneficial for symptomatic control in rheumatoid arthritis.[73]

Carotid artery intima-media thickness and carotid plaques are markers of cardiovascular health. Carotid plaque formation and intima-media thickening are positively correlated with increased duration of rheumatoid arthritis. Intima-media thickness is positively correlated with CRP concentration, which also happens to be predictive of 10 year mortality in rheumatoid arthritis patients. Auto-reactive T cells and TNF-α, which are common to atherosclerosis and rheumatoid arthritis, may also contribute to the co-existence of these diseases.[70] From these examples we see the overlapping, inflammatory, nature of cardiovascular disease and rheumatoid arthritis.

Current anti-inflammatory treatments for rheumatoid arthritis might reduce atherosclerotic risk. However, glucocorticosteroids tend to increase blood pressure, alter body fat distribution, and increase blood glucose, all which would worsen atherosclerosis.[1] Non-steroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors may not be a good choice for lowering cardiovascular risk either since both may increase cardiovascular risk.[1, 77] Hydroxychloroquine, chloroquine, and methotrexate (despite elevating homocysteine) might be useful because they demonstrate a variety of anti-platelet, anti-thrombogenic, and anti-inflammatory properties.[1] The risk to benefit ratio for reducing cardiovascular risk in rheumatoid arthritis patients using current anti-inflammatory therapies needs to be studied carefully. Non-traditional anti-inflammatory approaches that may improve atherosclerosis in rheumatoid arthritis include the use of folic acid, selective estrogen receptor modulators, statins, or B vitamins.[1]

1.1.2.2.4. Experimental arthritis

Experimental arthritis models have provided researchers with the ability to closely study rheumatoid arthritis. There are a number of experimental arthritis models. In the rat, collagen-induced arthritis, adjuvant arthritis, streptococcal cell wall-induced arthritis are common examples of arthritis models, and many others exist.[78] This section is primarily concerned with adjuvant arthritis since it is part of our experimental design.

Collagen-induced arthritis causes joint erosion because hyperimmunization leads to the development of auto-antigens to the animal's own collagen. Collagen-induced arthritis is similar to the human disease due to the presence of rheumatoid factor, inflammatory cell infiltration, persistent joint inflammation, synovial hyperplasia, and the animal is responsive to treatments that are normally successful in humans.[78] However, collagen induced arthritis causes the production of anti-collagen antibodies that are not always present in humans.[78] Adjuvant arthritis (AA) is similar to human disease due to the infiltration of inflammatory cells, persistent joint inflammation, synovial hyperplasia, and as with collagen-induced arthritis, the animal is responsive to treatments that are normally successful in humans. AA differs from rheumatoid arthritis in a few ways, including the absence of rheumatoid factor and the presence of extra-articular effects not normally observed in humans.[78] Streptococcal cell wall-induced arthritis is similar to rheumatoid arthritis as demonstrated by the infiltration of inflammatory cells, persistent joint inflammation, and synovial hyperplasia. However, streptococcal cell wall-induced arthritis does not result in the presence of rheumatoid factor in the circulation.

AA is achieved by injecting an animal with complete Freund's adjuvant. This causes elevations in pro-inflammatory mediators, and subsequently produces systemic arthritis.[13] Important for our studies, the adjuvant arthritis disease process is similar enough to rheumatoid arthritis that even cardiac changes are observed, and not just changes in joint structure or function. The Pre-AA model uses the same agents as AA, but studies are terminated before the animal begins to develop the painful symptoms of arthritis.

Complete Freund's adjuvant contains desiccated *Mycobacterium sp.* (dose-related) incorporated into an oil phase such as squalene. The oil helps prolong antigen exposure, and helps target the complete Freund's adjuvant to the lymphatic system. Bacterial components are taken up by phagocytic immune cells, including dendrocytes. This causes cytokine release by mononuclear phagocytes, including monophages. Subsequent to cytokine stimulation, the $CD4^+$ lymphocyte population becomes activated, differentiated, and expanded. The ensuing T_H1-skewing of T lymphocytes (which uniquely secrete IFN-γ), sets up a delayed-type hypersensitivity reaction to self-antigens. At first the self-antigens cause acute periarticular inflammation, but eventually bone tissue is involved.[13, 65] Symptoms of AA show up about 14 days after the injection of complete Freund's adjuvant. Measurable differences in inflammatory mediator concentration are present within 1 day. Ling and Jamali show in rats that circulating CRP is elevated by Day 1. By Day 3, circulating nitrite is elevated. By Day 6, circulating TNF-α is elevated.[79] A good rheumatoid arthritis animal model must have symptoms that are similar to that of humans. The model must also have an inflammatory profile that resembles humans with rheumatoid arthritis. CRP, IL-1, IL-6, TNF-α, and IFN-γ are all found in higher-than-normal concentration in both humans and animals with rheumatoid arthritis or rheumatoid arthritis-like conditions.[79-81]

Our interests in Pre-AA pertain to changes in the cardiovascular system, not changes in the musculoskeletal system. Adjuvant arthritis causes vast amounts of pain and suffering as a result of acute peri-articular inflammation. If one can eliminate these undesirable features of the animal model, yet still establish an altered cardiovascular system, then the experiments could be considered more humane. Being concerned with the therapeutic potency of cardiovascular agents, but not musculoskeletal effects, Ling and Jamali introduced the pre-adjuvant arthritis (Pre-AA, also known as Early-AA) model of inflammation.[79] The main benefit is that animals are only kept for 8-12 days. This prevents the development of excessive pain and discomfort in the rats. Importantly, cardiovascular adaptations to rheumatoid-like inflammation are observed. Therefore, this model offers a more humane approach to rheumatoid-like inflammation research.[79]

Pre-AA has now been shown to have three important similarities to adjuvant arthritis. Firstly, parallel pharmacokinetic changes occur in both AA and rheumatoid arthritis. This is demonstrated by a reduced verapamil free fraction (the portion not bound to plasma proteins) and reduced hepatic cytochrome P450 CYP1A and CYP3A content. Importantly, verapamil plasma concentrations are significantly elevated after both oral and i.v. doses.[79] Secondly, reduced cardiac responsiveness is demonstrated by reduced PR interval response to both propranolol and verapamil.[82, 83] Finally, Pre-AA results in T_H1 immune response skewing,[82] an important characteristic of rheumatoid and adjuvant arthritis.[14] Joint effects relating to collagenous changes or MMP activity are presently unknown in the Pre-AA rat model. The onset of AA occurs at approximately 14 days, but the specific day is species-dependent.

1.2. *Inflammation alters pharmacokinetic properties of drugs*

Inflammation can change the pharmacokinetic properties of drugs. Various mechanisms are responsible for this phenomenon, which the following sections will describe. Section 1.2.4 will describe the effect of inflammation on the pharmacodynamics and pharmacokinetics of various cardiovascular drugs.

1.2.1. Absorption

Due to convenience, oral dosing is preferred over other routes of administration. However, many variables can influence either the rate or degree of absorption, and hence making plasma concentrations unpredictable. The successful absorption of a drug product is determined by many variables including disintegration, dissolution, permeability, gastro-intestinal transit rate, gastrointestinal tract enzymatic stability, and drug stability in the gastric and intestinal juices.[84]

To reach the circulation, drug molecules must pass through the epithelial membrane of the intestinal tract. This process can occur passively by allowing drug molecules to pass through gap junctions (paracellular). Transcellular transport is either passive or carrier-mediated. Transcellular transport may also include vesicular, pore, or ion-pair transport. Of all the aforementioned drug absorption processes, passive transport is the most common.[85]

Active transport systems include carrier-mediated transport. Active transport is energy-consuming but usually highly specific (e.g. P-glycoprotein). Facilitated diffusion, which moves drugs along concentration gradients, does not require energy. These mechanisms are generally saturable, and require non-linear mathematics for process description.[85] When compared to those drugs with passive absorption, drugs that are absorbed by saturable mechanisms tend to have highly variable plasma concentrations.

Any process that alters the following may also alter the absorption profile of a drug: a) intestinal blood flow, b) gastrointestinal motility, c) stomach emptying time, d) gastric or intestinal pH, e) gut permeability, f) bile secretion, g) enzyme secretion, or h) gastro-intestinal flora.[85] For example, small intestinal mucosa is damaged by non-steroidal anti-inflammatory drug administration. This is shown to increase the permeability of Cr^{51}-EDTA, which normally does not traverse the small intestine.[86] Non-steroidal anti-inflammatory drug treatment increases drug permeation by damaging tight junctions that are involved in paracellular transport.[86] Also, inflammation can slow gastrointestinal motility, thereby reducing the absorption rate of drugs.

Inflammation itself can increase the permeability of the intestines. IFN-γ and TNF-α have been shown to increase permeability in vitro. Inflammatory gastro-intestinal diseases such as Crohn's disease can directly affect drug absorption because of localized inflammation. In Crohn's disease patients, elevated IL-12 concentrations and increased numbers of mast cells in the intestines, helps to enhance the permeability of macromolecules, a process which is important in Crohn's disease progression.[87, 88] From a clinical perspective, it may be important to consider that Crohn's disease patients are more likely than healthy subjects to have decreased gastro-intestinal transit time. This may limit drug absorption if the absorption window is small.

The potential for inflammation to interfere with normal absorption processes should be considered when conducting research on subjects with inflammatory diseases.

1.2.2. Distribution and protein binding

Drugs equilibrate between the free and bound state within the circulatory system. Unable to diffuse into peripheral tissues, the bound fraction is confined to the circulation. It is only the unbound portion of drug (the free fraction) that is able to leave the central vasculature and interact with pharmacologic targets, tissue binding proteins, and drug-metabolizing enzymes. It is the free fraction of the drug which equilibrates between the central and peripheral compartments. As the bound fraction of drug becomes higher there tends to be a restriction in the distribution of the drug, effectively reducing the volume of distribution.[85] Additionally, as protein binding increases, the ability to clear drug generally goes down. Protein binding can be influenced by various diseases, including inflammatory diseases or hepatic insufficiency.

Proteins that commonly bind drugs are α-1-acid glycoprotein, albumin, lipoproteins, and immunoglobulins. Free drug can also be up taken and sequestered by platelets and red blood cells. High levels of acidity in the lysozymes and granules of leukocytes and platelets can ion-trap basic drugs, sometimes in a stereoselective manner.[89] Hydroxychloroquine concentration is 7 -10 fold higher in whole blood than in plasma, largely due to high levels of uptake in leukocytes and platelets.[89] Binding primarily weak acids, albumin is the most common protein which contributes to protein binding. Albumin is found in higher concentrations in the plasma than in the interstitium. α-1-acid glycoprotein generally binds cationic basic drugs, and because the concentration of this protein is quite low it is more subject to saturable binding.[85]

Protein binding can also alter drug elimination. Highly protein bound drugs have low free fractions, effectively preventing drug molecules from entering hepatocytes where metabolism occurs, or from diffusing through glomeruli where drug excretion occurs. With regards to metabolism, protein bound drugs can be either restrictively or non-restrictively eliminated. If the drug rapidly equilibrates off of the protein then protein binding is essentially not restricting its metabolism. This situation occurs with propranolol, which is highly protein bound, but is not restrictively cleared.[85]

Elevated α-1-acid glycoprotein concentration found in inflammation can alter the protein binding of drugs. This happens after surgery, during infection, in chronic pain, with myocardial infarctions, in rheumatoid arthritis, and even in renal or hepatic disease.[90] Highly protein bound drugs are more likely to exhibit pharmacokinetic changes during inflammatory processes.[91]

As it turns out there are very few situations where changes in protein binding are actually clinically relevant. Practically speaking, changes in protein binding are only clinically relevant if the drug is administered intravenously, whether it is cleared by the liver, or not.[92] Changes in protein binding can influence pharmacokinetic parameters, but it tends to cause few problems.

Drug distribution may be influenced by P-glycoprotein, a 170 kDa phosphoglycoprotein of the ATP-binding cassette family. It transports molecules across cell membranes, and during inflammation its function and expression decrease. This can impact drug distribution into cells, or even into the brain, possibly altering drug toxicity or efficacy.[93]

P-glycoprotein expression in the heart may be cardioprotective if it limits the uptake of cardiotoxic agents. Idarubicin is a cardiotoxic chemotherapeutic agent. Verapamil, a potent P-glycoprotein inhibitor,[94] should theoretically lead to increases in idarubicin concentration in the heart by preventing P-glycoprotein from pumping idarubicin out of cardiac tissue. This is a highlight in the controversy surrounding the P-glycoprotein literature as it pertains to cardiac tissue. In 2002 Weiss and Kang concluded that the P-glycoprotein inhibitor, verapamil, when administered to isolated heart, enhances myocardial uptake of idarubicin.[95] The same group in 2007 modified their conclusion based on an experiment which showed that the P-glycoprotein inhibitor[96] amiodarone did not alter verapamil concentrations in the heart. It could be that the levels of P-glycoprotein expressed in the heart are just too low to be of significance with these specific compounds. It could be that inflammation does not play a major role in altering P-glycoprotein levels in the myocardium. Neither tachycardia-induced heart failure in dog, nor ischemic heart disease in human affect P-glycoprotein levels in myocardium.[97, 98]

1.2.3. Metabolism

Inflammation, in general, reduces drug metabolism, and hence clearance. This is true regardless of the source of inflammation, whether it be from septic shock, *Streptococcus pneumoniae*, influenza, or influenza vaccine.[91] Non-infectious inflammatory processes also reduce metabolic activity in a broad range of conditions that include burns, cancer, mechanical tissue damage, and auto-immune diseases, such as rheumatoid arthritis.[99] Drugs with a high hepatic extraction ratio are at highest risk of pharmacokinetic changes after the induction of inflammation,[91] however changes can occur with low extraction ratio drugs as well. For example, influenza A increases theophylline plasma concentrations in children taking theophylline for asthma.[100] When the clearance of a high extraction ratio drug is reduced by a large enough degree, a drug may be converted from a high extraction into either a mid to low extraction drug. In inflammation, large reductions in clearance can occur by either reducing the unbound fraction of drug (if the drug is bound by α-1-acid glycoprotein) or by reducing intrinsic clearance.[101]

Inflammation has a non-specific inhibitory effect on cytochrome P450 drug-metabolizing enzymes, the enzymes that account for a majority of drug metabolism. Nitric oxide, which is highly toxic to bacteria likely on account of peroxynitrite, is synthesized in large amounts during inflammation, especially by the liver. Nitric oxide production is stimulated by cytokines such as IL-1 and TNF-α. Elevated concentrations of nitric oxide are able to decrease drug-metabolizing enzymatic activity and reduce total liver enzyme content.[100]

The majority of cytochrome P450 enzymes are constitutively expressed. Inflammation can reduce gene transcription, increase the turn-over of RNA and protein, or change the protein translation rate. Not all of the cytochrome P450 enzymes are affected to the same degree, nor are they affected for the same duration, and so each drug metabolizing enzyme must be considered individually when evaluating the impact of inflammation.

The effect of inflammation on the expression and function of phase II metabolic reactions is less well known. Pig hepatocytes, in the inflamed state, have a reduction in uridine diphosphate glucuronyltransferase activity, effectively reducing glucuronidation.[102] Also, the glucuronidation of ketoprofen in rats is reduced in the presence of adjuvant arthritis.[103] Therefore, drug metabolism in general is decreased with inflammation, though exceptions occur. Some of these exceptions include the up-regulation of CYP2A6 in Hepatitis B and C, renal failure, the up-regulation of CYP4F16 with lipopolysaccharide administration, and the up-regulation of CYP4A in particulate matter administration.[99]

1.2.4. Cardiovascular drugs – examples

In addition to the ability of inflammation to alter the pharmacokinetic property of drugs, inflammation can also alter the pharmacodynamic property of a drug. Decreased potency or efficacy of cardiovascular drugs is of particular concern since it may undermine cardiovascular risk reduction strategies. Various drug classes are represented in this section, but propranolol, a β-blocker, receives the most amount of attention since this is the drug being used in the experiments of this thesis.

The PR interval is defined as the duration of time between atrial activation and ventricular activation. Prolonging the PR interval indicates slowed conduction velocity. Reducing conduction velocity can be advantageous in certain disease processes, as it is with supraventricular tachyarrhythmia. Careful examination of the human ECG waveform Figure 1-1, shows that the label 'PQ-interval' would more aptly describe the PR interval, however, 'PR interval' remains the convention.[104] Figure 1-2 below shows a typical PR interval in the male Sprague-Dawley rat.

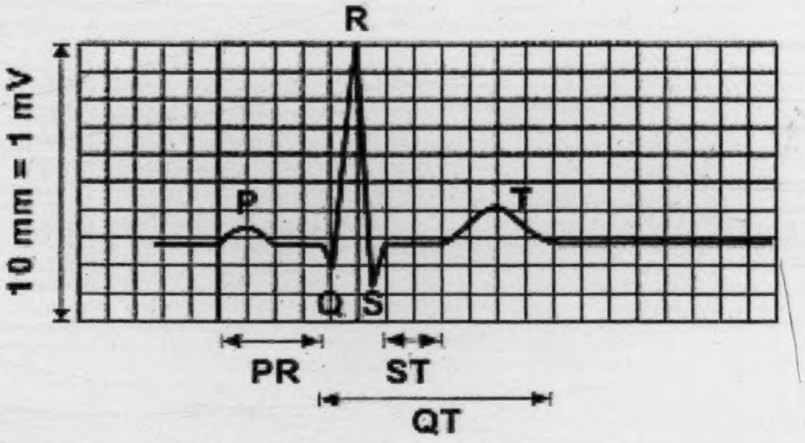

Figure 1-1. Electrocardiographic representation of the PR interval in human.[105]

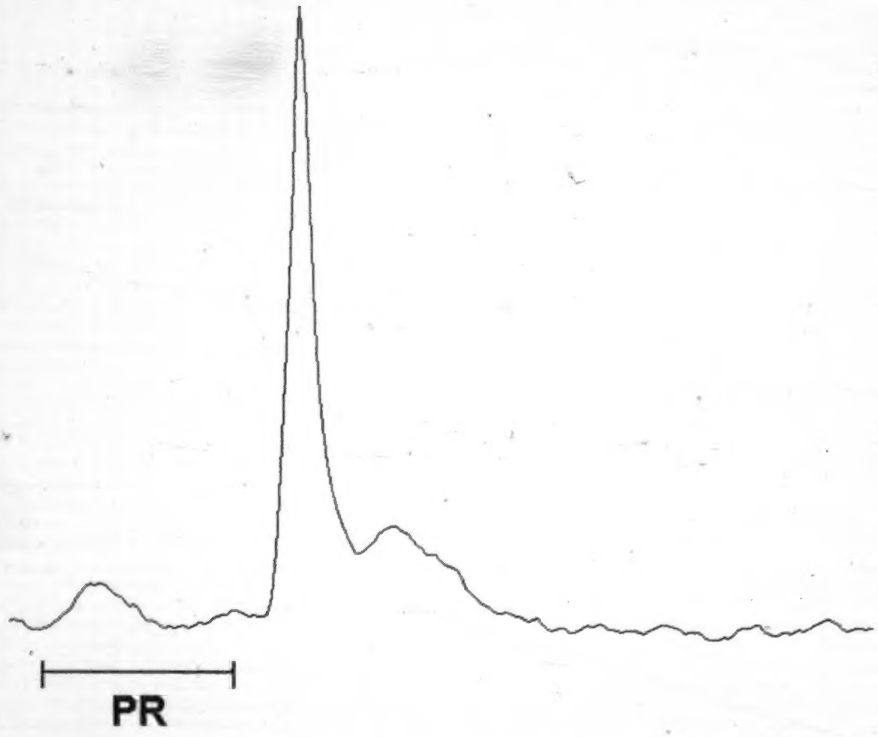

Figure 1-2 Electrocardiographic representation of PR interval in rat.

1.2.4.1. Propranolol

Propranolol is a β-AR blocker which has been widely used in the treatment of hypertension, angina, acute myocardial infarction, supraventricular tachyarrhythmia, and ventricular tachyarrhythmias.[106] Propranolol is 90-95% protein bound, primarily to α-1-acid glycoprotein, and has a half-life of 1.5-3.0 hours. Propranolol has a very high extraction ratio with single oral dosing, whereas multiple dosing diminishes the hepatic extraction ratio due to enzyme saturation. These attributes contribute to a twenty-fold variation in propranolol plasma concentration after repeated oral dosing, even within the same patient.[107]

The first report of a human inflammatory condition causing elevated propranolol concentrations was in 1976.[108] Crohn's disease patients were observed to have propranolol concentrations 5 times higher than healthy counterparts.[108] Within a few years, pneumonia, ulcerative colitis, systemic lupus erythematosus, and rheumatoid arthritis (all diseases that demonstrate inflammatory processes) were also characterized as having elevated propranolol concentration. Importantly, the degree of inflammation, as measured by erythrocyte sedimentation rate, was correlated with the degree of propranolol plasma concentration elevation.[109]

Large increases in cardiovascular drug plasma concentration in inflammatory diseases inspired Schneider et al. to investigate the mechanisms of increased concentrations in humans using metoprolol, oxprenolol, and propranolol.[110] They postulated that elevated drug concentration in inflammation was due to a number of possible explanations, including increased absorption, reduced metabolism, or increased plasma protein binding. Metoprolol, oxprenolol, and propranolol are all highly absorbed, so variations in absorption could not contribute to altered plasma concentration of drug. Both metoprolol and propranolol are highly metabolized. Metoprolol has low protein binding whereas propranolol has high protein binding. Since metoprolol concentration was not elevated in the presence of inflammation whereas propranolol concentration was elevated, Schneider et al. concluded that it must have been protein binding that contributed to the elevation in plasma concentration of particular cardiovascular drugs. They did not think that reductions in metabolic function were responsible for elevated cardiovascular drug concentrations.

Authors other than Schneider et al. have also concluded that excessive protein binding in inflammation increases propranolol concentration.[111, 112] However, since propranolol is non-restrictively cleared,[107] this seems unlikely. Perhaps the ultra-high concentration of propranolol after oral dosing causes saturation of metabolizing enzymes in the liver upon first pass. Saturation would therefore allow a higher fraction of drug to reach the general circulation. Knowing that excessive concentrations of the inflammatory mediator nitric oxide can reduce CYPP450 activity and content,[100] high concentrations of nitric oxide may reduce the capacity of the metabolic enzymes. This may not be clinically relevant after intravenous propranolol dosing because the terminal half life does not change. During the first pass effect of oral propranolol dosing, inflammation may cause the extraction ratio to switch from high to medium or low, and therefore increase bioavailability.

As with humans, propranolol concentration in the plasma of inflamed rats is significantly elevated after oral dosing. In the adjuvant arthritis and Pre-AA rat models of inflammation, the area under the curve (AUC) for propranolol concentration after oral dosing in plasma can go up by 13-16 fold for R-propranolol, and by 4-14 fold for S-propranolol.[82, 113-115] Surgery and endotoxin-induced inflammation also significantly elevate propranolol concentration. After laparotomy, for example, orally dosed rats have a propranolol concentration that is two-fold higher than in control rats, possibly due to increases in α-1-acid glycoprotein binding. Endotoxin-induced inflammation also elevates propranolol concentration in the rat after oral dosing.[116] In contrast to oral dosing, intravenous dosing is not subject to first-pass metabolism and therefore this problem is avoided.[113]

When comparing control subjects to inflamed subjects, one might expect inflamed subjects to have heightened pharmacologic responses due to elevated plasma propranolol concentration. To the contrary, and despite having many fold increases in blood concentration, the pharmacodynamic response to propranolol is diminished in inflamed rats. Guirguis and Jamali 2002 have shown in rats that adjuvant arthritis elevates both unbound and bound total propranolol concentrations, and importantly that PR interval responsiveness is diminished.[114] Yasuhara et al. 1985 have shown in rats undergoing surgery that blood concentrations of propranolol are two-fold higher, yet there is diminished potency for tachycardia reduction.[116] However, in this instance, when accounting for unbound propranolol this property is no longer apparent.[116]

Since propranolol is given as a racemic mixture, consideration for individual enantiomers is important. S-propranolol is more potent at reducing blood pressure, by approximately 100 times, than is the R enantiomer.[113] In non-inflamed rat, the R- to S-propranolol ratio ranges from 4:1 to 6:1, whereas in adjuvant arthritis and Pre-AA it ranges from approximately 11:1 to 14:1.[82, 113, 114] The difference between R- and S-propranolol concentration is likely due to differential presystemic and systemic clearance.[113] Similarly, in endotoxin-induced inflammation, the R-enantiomer of propranolol is stereoselectively increased compared to the S-enantiomer.[117]

1.2.4.2. Verapamil

Verapamil is an L-type calcium channel antagonist that is used in the treatment of angina, cardiac arrhythmias, and cardiomyopathies. It reduces cardiac electrical activity and myocardial contraction by reducing intracellular calcium concentration.[118] Mayo et al. 2000 have shown that patients with rheumatoid arthritis have lower verapamil response despite having significantly higher bound drug concentration, though similar unbound AUCs were found for the more active S-enantiomer.[4] They have also shown that verapamil concentration is positively correlated with plasma nitric oxide. The authors attributed reduced drug clearance to elevated protein binding, altered hepatic blood flow, or reductions in CYP activity.[4] This information gained from this study may be relevant to the inflammation associated with age and obesity, which can also reduce the pharmacodynamic responsiveness to verapamil.[91, 119, 120]

Sermsappasuk et al. 2007 have shown in the isolated hearts of lipopolysaccharide-treated rats that inflammation diminishes the negative inotropic effects of verapamil.[121] Isolated hearts subjected to a simulated bolus dose of verapamil were perfused with Krebs-Henseleit buffer containing identical 0.1% bovine serum albumin concentration as the control group. Therefore, changes in response could not have been due to differences in protein binding. Since differences in heart outflow verapamil concentrations were much smaller than differences in cardiac response to the drug, it was concluded that receptor density or binding is reduced. Fever, which may be used as a surrogate marker for the degree of inflammation, was negatively correlated with verapamil E_{max}. This suggested that inflammatory mediators, either directly or indirectly, reduced the pharmacodynamic response.

IFN-induced inflammation in rats, at 12 and 2 hours before dosing, is also associated with an increased concentration of verapamil, however not with changes in protein binding.[122] Importantly, PR interval (dromotropic) responsiveness is also reduced. While interferon does not elevate verapamil concentration, it does decrease the dromotropic response to the drug. Ling and Jamali 2005 showed in the Pre-AA rats that verapamil clearance is reduced in both orally and intravenously dosed rats.[79] Importantly, the authors were able to show that inflammation reduces CYP3A/1A content and verapamil free fraction.

As with propranolol, verapamil is a mixture of two enantiomers. The S enantiomer of verapamil has a volume of distribution approximately 2.3 fold higher than the R enantiomer.[123] Plasma protein binding is higher with the R enantiomer of verapamil, likely restricting its distribution into deeper tissues. Additionally, S verapamil is cleared faster than R verapamil, contributing to substantially higher levels of R-verapamil in plasma.[123] Oral dosing results in bigger differences in verapamil enantiomer concentration when compared to intravenous dosing, because the rate of drug delivery to the liver influences pharmacokinetic stereoselectivity.[123]

1.2.4.3. Sotalol

R-sotalol is both a β_1-AR blocker and potassium channel blocker. The pharmacologic activity of the S enantiomer is, however, restricted to potassium channel blocking. Sotalol is actively secreted by the kidneys, and has limited plasma protein binding.[123] It is important to note that, unlike with propranolol and verapamil, the ratio of S to R sotalol AUC approximates 1, even in the presence of inflammation.[123, 124]

Kulmatycki et al. 2001 performed an experiment that investigated the effect of acute and chronic inflammation on the pharmacokinetics and pharmacodynamics of sotalol. They induced acute inflammation in rats by administering interferon α-2a at 12 and 3 hours before sotalol response testing.[125] They induced chronic inflammation (adjuvant arthritis) by injecting *Mycobacterium butyricum* in squalene into the lymphatic tissue of the tail-base. Both acute and chronic inflammation reduced the degree of QT interval response to sotalol racemate. In acutely inflamed rats, racemic sotalol, R-sotalol or S-sotalol administration elicited smaller QT interval responsiveness. In chronically inflamed rats racemic sotalol induced a smaller PR interval prolongation. In acutely inflamed rats, racemic sotalol and R-sotalol elicited a smaller PR interval response. Since the pharmacokinetics of sotalol were unchanged by inflammation, reduced drug response was likely related to reduced β_1-AR and potassium channel sensitivity, possibly by altered receptor density, receptor configuration, or second messenger systems.[124] By showing that anti-TNFα therapy with infliximab reduced nitric oxide and TNFα concentrations, and that anti-TNFα therapy restores QT and PR interval response, Kulmatycki et al. 2001 demonstrated the in vivo link between mediators of inflammation and reduced cardiac pharmacodynamic response.

1.2.4.4. Angiotensin II type 1 receptor antagonists

Further investigation into the effects of inflammation on the pharmacokinetics and pharmacodynamics of cardiovascular drugs has revealed two notable exceptions. Losartan and valsartan are angiotensin II type 1 receptor antagonists, which are effective in the treatment of hypertension, renal failure and congestive heart failure.[126]

Losartan, a prodrug, must be converted to the metabolite E3174 in order to elicit a pharmacodynamic response. In inflammation, metabolic conversion of losartan to its active metabolite E3174 might be impaired. Single dose losartan pharmacokinetics in rheumatoid arthritis patients caused a significantly reduced area under the concentration curve for E3174 when compared to controlled rheumatoid arthritis patients and healthy subjects.[127] This was thought to be indicative of reduced metabolic conversion, despite the fact that losartan concentrations were not significantly different.[127] Despite lower concentration of the active metabolite E3174, no reductions had been noticed in pharmacodynamic response as measured by mean arterial pressure, systolic blood pressure, diastolic blood pressure, pulse, cardiac ejection time or other cardiovascular measurements. The authors of this study, Daneshtalab et al. 2006, suggested that inflammatory mediators might increase the responsiveness of angiotensin II type 1 receptors, which is contrary to the effects of inflammation on β_1-ARs, L-type calcium channels, or potassium channels.

In a study similar to that of losartan, healthy subjects versus controlled and active rheumatoid arthritis were examined for differential effects of rheumatoid arthritis on valsartan pharmacokinetics and pharmacodynamics.[128] Inflammation associated with arthritis did not cause any changes in valsartan plasma concentration. This may have been because inflammation does not reduce renal function like it does liver function and because valsartan is only 20% metabolized. However, valsartan administration in rheumatoid arthritis patients did show a non-significant trend towards increased hypotensive effects.[128] These findings suggest that angiotensin II type 1 receptors may be upregulated in inflammation. These findings were consistent with the findings of the above losartan study.[128]

1.3. *The β_1-adrenergic receptor*

When investigating the causes of altered propranolol response in inflammation, having a full understanding of the β_1-AR is critical. The complexity surrounding drug-receptor interactions, receptor signal transduction, and receptor regulation, implies that myriad potential causes exist for reduced propranolol response during inflammatory processes. The section ahead characterizes the β_1-AR, explores the factors that alter β_1-AR functionality, and analyses the β_1-AR in the context of rheumatoid arthritis.

Inflammation is pivotal in the development of rheumatoid arthritis, having direct effects not only in joints, but also in various physiological systems or anatomical sites. The heart is one such location that inflammation can have serious consequences. In the heart, impaired signaling and function is observed during inflammatory states with the β_1-AR. This section conceptualizes β_1-ARs in terms of normal receptor signaling and regulation, then focuses on the effects of sympathetic activity, inflammation, and rheumatoid arthritis. Finally, consideration is given to the potential use of statins, angiotensin II type 1 receptor blockers, and β_1-AR blockers in reducing the rheumatoid arthritis patient's heightened risk of cardiovascular disease.

1.3.1. Overview of the β1-adrenergic receptor

The cardiovascular system is partly regulated by the sympathetic and parasympathetic nervous systems, targeting both adrenergic and muscarinic receptors, respectively. Three α_1-adrenergic receptors, three α_2-adrenergic receptors, and three β-ARs have been discovered,[129] all of which are expressed in varying amounts in the heart. The putative β_4-AR is now thought to be a polymorphic state of the β_1-AR that happens to be propranolol resistant.[130-133]

Of the various receptors in the heart, the β_1-AR is the most prominent and influential on cardiac function.[134, 135] The β_1-AR influences chronotropy, inotropy, lusitropy, dromotropy, and the regulation of blood pressure.[114, 136] β_1-ARs are also involved in adipose tissue lipolysis and memory development.[137]

Cardiac β_1-ARs respond to locally secreted norepinephrine, to circulating catecholamines (released from the adrenal glands),[138] and to exogenously administered catecholaminergic drugs. Since β_1-ARs are primarily a postsynaptic receptor, norepinephrine release from nerve synapses is the biggest contributor to its stimulation.[138, 139] Under normal physiological conditions, circulating norepinephrine does not influence cardiac function because high concentrations are not normal except during heavy exercise or pathological conditions.[140]

β_1-ARs are found in highest density in heart, lung, testis, kidney, spleen, thyroid, pineal gland, and cerebral cortex.[139, 141] The cardiac ventricles have a β_1-AR: β_2-AR ratio of approximately 75%: 25%, and in cardiac atria of approximately 65%: 35%.[129] Atria have higher levels of β_1-ARs than do ventricles,[142] and the highest density within the atria is at the sino-atrial node; the pacemaker of the heart.[129] The highest β_1-AR density in the ventricles is within the subendocardial myocytes.[143]

Cell membrane arrangement of the β_1-AR, a G-protein-coupled receptor, is complex. G_s, the stimulatory G-protein, and adenylate cyclase are both coupled to the third intracellular loop of β_1-ARs in a proline-rich area. This proline-rich area, which does not exist in the β_2AR, reduces the affinity of the G_s-adenylate cyclase complex to the β_1-AR, thereby imparting negative modulation when compared to the β_2-ARs.[143] The reduced affinity of the G_s-adenylate complex to the β_1-AR explains why, physiologically, the β_2-AR elicits stronger stimulatory responses than does the β_1-AR. Other notable features of the β_1-AR are summarized in Table 1-3.

Evidence shows that G-protein-coupled receptors are not randomly distributed proteins, but rather they are compartmentalized. β_1-ARs co-localize with its major down-stream effector molecule adenylate cyclase-6 in caveolin-rich domains in rat cardiac myocytes.[144] In humans, adenylate cyclase-6 is the most highly expressed of the four mammalian adenylate cyclase subtypes.[145]

Table 1-3. Notable features of the β1-Adrenergic Receptor

Location	Feature	Function
Asn15 – (Extracellular N-terminus)	N-Glycosylation	Regulation of surface expression and dimerization[146, 147]
C-Terminal	GRK-mediated phosphorylation	Desensitization[136, 138]
Residue 389 (Intracellular C terminus)	Glycine/arginine polymorphism	May be correlated to disease, especially in black people[148, 149]
Residue 49 (Extracellular N-terminus)	Serine/glycine polymorphism	May increase the impact disease[138, 150, 151]
Residues 131 and 209	Disulfide bridge	Receptor rigidness[150]
Second extracellular loop	Binding site for IgG β-AR antibodies	Stabilization of active receptor configuration. Induction of heart failure[150]
Serine/Threonine-rich cytoplasmic tail (10 residues)	GRK-mediated phosphorylation of occupied receptors	Desensitization[152, 153]
Third intracellular loop	PKA*-mediated phosphorylation	Desensitization[138, 150, 151]
Third intracellular loop	Proline rich sequence	Negative modulation of β_1-AR binding with G_s and adenylate cyclase[143]

*protein kinase A

1.3.2. Control and signaling of the β1-adrenergic receptor

The β_1-AR is an intermediate which links adrenergic molecules of the sympathetic nervous system to specific intracellular processes. The β_1-AR helps to convert a neurological or chemical signal into an elicited response. Agonist binding of the β_1-AR creates a ligand – receptor – G_s protein complex, which causes the exchange of one molecule of guanosine diphosphate for one molecule of guanosine triphosphate.[154] Guanosine triphosphate binding instigates the dissociation of β_1-AR-attatched G_s into $G_{\alpha s}$-guanosine triphosphate and $G_{\beta\gamma}$. Subsequently, the $G_{\alpha s}$ protein complexes with guanosine triphosphate, and finally activates adenylate cyclase. Adenylate cyclase catalyses the formation of intracellular cAMP, which is an important second messenger.[136, 138] The other half of the G_s complex, the βγ subunit, acts as a membrane trap for the G-protein-coupled receptor kinases (GRKs), which facilitate the phosphorylation and subsequent desensitization of the β_1-AR.[154] This is an example of a homologous desensitization system.

1.3.2.1. Calcium concentration and L-type calcium channels are regulated by β1-ARs

β_1-ARs and L-type calcium channels are functionally linked by a second messenger system: namely cAMP. This enables β_1-AR simulation to influence calcium concentrations in the cardiomyocytes, and therefore exert partial control over cardiac electrical conduction and cardiac muscle contraction. The coupling of the L-type calcium channel to β_1-ARs is stronger when compared to β_2-ARs.[155] After simulation by the sympathetic nervous system, β_1-ARs activate adenylate cyclase, and consequently elevate intra-cellular cAMP concentration.[135] The main downstream effect of increased cAMP is activation of protein kinase A. Protein kinase A is a pivotal protein that alters calcium concentration via the phosphorylation of: a) L-type Ca^{2+} channels, b) Na^+/K^+ ATPase, which increases Ca^{2+} efflux via Na^+/Ca^{2+} exchange, c) ryanodine receptors, and d) phospholamban.[129, 135, 138] These listed effects are the main mechanisms involved in changing calcium concentration in myocardium upon β_1-AR stimulation.[156] Elevated calcium concentration subsequently increases cardiomyocyte inotropy and chronotropy.[135]

After protein kinase A phosphorylates phospholamban, the phospholamban is less able to interact with, and inhibit, sarcoendoplasmic reticulum Ca^{2+}-ATPase. Sarcoendoplasmic reticulum Ca^{2+}-ATPase sequesters calcium by pumping it into the sarcoplasmic reticulum, which helps cease contraction and increases the availability of calcium for subsequent contractions.[157] Protein kinase A also phosphorylates troponin I, which helps make myofilaments less sensitive to calcium.[158] The phosphorylation of both sarcoendoplasmic reticulum Ca^{2+}-ATPase and troponin I, by protein kinase A, contributes to increased contraction-relaxation cycling, and hence facilitates cardiac lusitropy.[158]

1.3.3. Regulation of the β1-adrenergic receptor

Short-term regulation of the β_1-AR occurs by receptor desensitization, uncoupling, and internalization. Over the long-term, the β_1-AR is regulated by mRNA degradation and altered gene expression.[138] Located on human chromosome 10q24-q26, the β_1-AR has a 5' flanking promoter region of approximately 3 kb.[129, 139] The receptor is under active basal promoter activity, meaning there normally is a continual mRNA renewal process.[159] The primary promoter is at -444 to -360 to the start site, though multiple transcription initiation sites also exist at -289 to -261.[139] Other positive and negative regulatory regions spanning the -3813 to -444 range help modify transcriptional activity.[134, 139, 159, 160]

Glucocorticoids, sex hormones, and thyroid hormones are all involved in the regulation of β_1-AR transcription.[160-162] Glucocorticoids may increase β_1-AR expression, in part, by interacting with a critical partial glucocorticoid response element.[160, 161] However, there are conflicting literature results that demonstrate glucocorticoid administration causes either increased or decreased effects on β_1-AR expression, depending on the study.[163] Progestin and estrogen exert negative control on the expression of β_1-ARs. The observation that sex hormone deficient rats express markedly more β_1-ARs, supports this claim.[134] In contrast, thyroid hormones have a marked positive effect on both the transcription and translation of β_1-ARs.[160, 164]

N-glycosylation of G-protein-coupled receptors, in general, results in a full range of functional changes. For example, oxytocin receptors undergo no change in receptor function following N-glycosylation, whereas thyrotropin receptors have complete abolishment of function.[137] N-glycosylation of β_1-ARs at Asn15 is highly influential; N15A mutation prevents N-glycosylation, resulting in less dimerization, less cell surface expression, and less isoproterenol response as gauged by cAMP production. Normal cell surface expression of the β_1-AR is therefore partly dependent on N-glycosylation-assisted dimerization.[137, 165]

1.3.3.1. Homologous desensitization

Desensitization of the β-ARs can serve as a protective mechanism by preventing the deleterious effects of chronic adrenergic stimulation, which in the short term improves cardiac function, but eventually confers damage to the myocardium.[136] Homologous phosphorylation of β_1-ARs is mediated by G-protein-coupled receptor kinases (GRK). The GRKs included GRK-1 (aka rhodopsin kinase), GRK-2 (aka βARK1), GRK-3 (aka βARK-2) and GRK-5.[154] GRKs are attracted to the β_1-AR by membrane-attached βγ subunits of the dissociated G_s protein.[136, 166] This process occurs with agonist-occupied receptors and causes phosphorylation of numerous serine and threonine residues that reside in the β_1-AR carboxy-terminus.[136, 138]

Subsequent to β_1-AR phosphorylation by GRKs, the β_1-AR is inactivated by either β-arrestin-1 or β-arrestin-2.[154] Consequently, β-arrestin deficiencies enable heightened isoproterenol contractile response.[136, 138] β_1-AR phosphorylation by GRKs also reduces isoproterenol affinity to β_1-ARs.[167] Although β_1-AR are somewhat less sensitive to agonist-stimulated regulation compared to the β_2-AR,[134] phosphorylation- and β-arrestin-assisted uncoupling of G_s both diminish adenylate cyclase activity, and therefore the overall activity of the β_1-AR.[154, 167]

1.3.3.2. Heterologous desensitization

In contrast with homologous phosphorylation and desensitization, the process of heterologous phosphorylation of β_1-ARs is not dependent on ligand binding. Rather, heterologous desensitization is cAMP-dependent, and it leads to widespread cellular inactivity, including diminished strength of β_1-AR signal transduction.[138] Heterologous desensitization is mediated by phosphorylated protein kinase A. GRK-mediated and phosphorylated protein kinase A-mediated heterologous desensitization processes contribute equally to β_1-AR desensitization.[135] GRK-2 concentration is, in part, controlled by cAMP concentration, and therefore the two desensitization processes may be in a state of cross-talk.[167]

When β_1-ARs are chronically under-stimulated, hypersensitization may occur.[168] Selective β_1-AR blockers, atenolol or metoprolol included, cause chronic understimulation of β_1-ARs, and hence increasing β_1-AR density.[151] This observation makes it easier to understand why some patients experience rebound hypertension upon β-blocker discontinuation. Blockade of β-ARs reduces the sympathetic to parasympathetic activity ratio in the heart.[169] This ratio is assessed performing a power spectral analysis of heart rate,[170] and is a useful indicator for arrhythmia risk.

1.3.3.3. Internalization

Agonist-mediated β_1-AR internalization is important for β_1-AR downregulation.[138, 151, 154, 166] The exact process of β_1-AR internalization appears to be dependent on the method by which the receptor has been phosphorylated.[166] Two pathways are used in the internalization process. First, GRK-mediated phosphorylation uses β-arrestins for receptor internalization by binding to clathrin-associated molecules. Subsequently, ligand-bound receptors are pulled towards pre-formed clathrin coated pits.[137] In the second pathway protein kinase A-mediated phosphorylation causes β_1-AR internalization via the caveolae pathway.[151] The contribution of each pathway to β_1-AR internalization is approximately equal, and additive, at high agonist concentrations. However, at low β_1-AR agonist concentrations, the clathrin-coated pit pathway predominates via GRK-mediated phosphorylation.[151]

1.3.3.4. mRNA turnover

Alteration in the mRNA turnover rate of β_1-ARs is another means of controlling receptor density. β_1-ARs have adenine/uracil-rich elements within 3'-untranslated regions that influence β_1-AR transcript degradation rates and therefore mRNA levels.[165] In rats, this process is agonist-mediated. Isoproterenol administration causes an increase in β-adrenergic receptor mRNA-binding protein.[165] β-adrenergic receptor mRNA-binding protein is comprised of two proteins, HuR and heterogeneous nuclear ribonuclear protein (hnRNP).[171] HuR binding to β_1-AR mRNA shortens the β_1-AR mRNA half-life.[165] Another protein that binds to β_1-AR mRNA is AUF1. AUF1 destabilizes β_1-AR mRNA, and it has been shown that high levels of AUF1 in human cardiac failure are associated with elevated norepinephrine.[172] The mRNA binding proteins influence pharmacodynamic responses.

cAMP-mediated downregulation of β_1-ARs occurs in the absence of internalization, and closely follows β_1-AR mRNA levels.[166] This is likely because the mechanism behind cAMP-induced downregulation is related to the interaction of the inducible cAMP early repressor with the cAMP response element in the promoter region of the β_1-AR.[166] The reduction in β_1AR mRNA, in this case, is delayed by hours because of the reliance on slow basal mRNA turnover following the reduction in transcription;[136] after all, β_1-AR mRNA has a 20 hour half-life.[165] Therefore, the cAMP-protein kinase A pathway causes reductions in β_1-AR receptor density by causing internalization via the caveolae pathway (see 1.3.3.3) and repression of mRNA translation.

1.3.3.5. Regulation of β1-adrenergic receptor in vivo

The expression of GRK-2 and β_1-AR occurs in a reciprocal manner. Ping et al. 1995, have shown that chronic antagonism of β_1-ARs in pigs receiving 0.2 mg/kg daily of bisoprolol (a selective β_1-AR blocker) causes a significant decrease in myocardial GRK-2 expression.[167] That is, reduced stimulation of β_1-

ARs causes less expression of its inhibitor (GRK-2). As expected, atenolol (a selective β_1-AR blocker), causes a significant decrease in myocardial GRK-2 expression, as was found with bisoprolol.[173] Atenolol administration also increases β-AR expression, in a manner reciprocal to GRK-2 expression.[173] In contrast, stimulation of β-ARs with isoproterenol in mice causes an approximate 2-fold *up-regulation* of GRK-2 expression and a reciprocal *downregulation* of β-AR expression by one-half.[173]

Compared to wildtype mice, transgenic mice that over-express GRK-2 experience dampened cardiac contractility with isoproterenol stimulation, attenuated adenylate cyclase activity, and reduced functional coupling of β_1-ARs.[174] As expected, transgenic mice that over-express the inhibitory protein GRK-2 have increased cardiac contractility, even in the absence of isoproterenol.[174] In dogs, the ventricular pacing-induced congestive heart failure model reduces β_1-AR density and adenylate cyclase activity.[175] Myocardial infarction also decreases β_1-AR density.[176] Overall, this again demonstrates the balance between GRK-2 and β_1-ARs in the myocardium.

Pro-inflammatory cytokines decrease cardiac function.[177, 178] Ex vivo, hearts from rats that are subjected to the septic shock model demonstrate a reduced ability to elevate cAMP upon isoproterenol administration.[179] Also, application of TNF-α to rat cardiomyocytes inhibits L-type Ca^{2+} channel current and contractile Ca^{2+} transients. This is relevant to the β_1-AR because the L-type Ca^{2+} channel is an effector protein in the β_1-AR signal transduction pathway. [180] Therefore, inflammation can alter β_1-AR signaling, and may be considered influential in the many inflammatory diseases that affect the heart, and in particular those that stimulate sympathetic tone.[181, 182] Examples include myocardial infarction, congestive heart failure, cardiac ischemia/ reperfusion injury, rheumatoid arthritis, and adjuvant arthritis.[31, 181, 183-186]

1.3.4. Polymorphisms of the β_1-AR

Of the 18 known β_1-AR single nucleotide polymorphisms only 7 lead to amino acid substitutions.[149] An adenine to guanidine single nucleotide polymorphism at nucleotide 145 (c145A>G) results in a Ser49 to Gly49 (Ser49Gly) substitution in the extracellular domain of the β_1-AR. African-Americans, Chinese, and Caucasians all have similar rates of mutation.[147] This mutation is associated with accelerated agonist-mediated downregulation because reduced Asn15 N-glycosylation enhances the internalization and degradation of the β_1-AR.[187] Congestive heart failure patients having the Gly49-β1 genotype, whether homo- or heterozygotic, have 23% mortality 5 years after diagnosis, compared to 46% mortality for patients with the Ser49 genotype. This supports the hypothesis that the Gly49 variant is cardioprotective in congestive heart failure.[149, 188]

The result of a c1165G>C single nucleotide polymorphism, where guanidine is replaced with cytosine, is that Arg becomes substituted with Gly at amino acid 389 (Arg389Gly).[147, 189] Affecting the intracellular portion of the β_1-AR, the Arg389 variant has enhanced adenylate cyclase coupling,[189] and greater basal and isoproterenol-stimulated adenylate cyclase activity.[149] Fat cells and metabolism are, in part, regulated by the β_1-AR. In human fat cells, catecholamine-induced lipolysis is not known to be influenced by the Arg389Gly polymorphism.[190] Remaining in isolation, the β_1-AR Arg389Gly polymorphism has no affect on obesity in a 24 year longitudinal study. However, when β_2-AR polymorphisms are taken into account, is has been shown that men with Gly/Gly genotypes for both β_1- and β_2-ARs have a significant 0.6%/year increase in body mass index from childhood to adulthood when compared to non-Gly/Gly men.[191] Although the Arg389 single nucleotide polymorphism is not associated with cardiovascular events *per se*, it may also be related to increased blood pressure[188, 192] and increased β_1-AR-blocker sensitivity.[193, 194] This is an example of a synergistic gene-gene interaction that has important effects on human health.

In addition to the Gly/Gly phenotype, the Arg389 phenotype of the β_1-AR becomes important when the patient has a co-existing α_{2c}-AR deletion mutation. Synergistic polymorphism between Arg389 of the β_1-AR and the deletion genotype α_{2c}-AR-Del322–325 is strongly correlated to disease. Black people with double homozygous Arg389 / α_{2c}-Del322-325 have a 10 times greater risk of developing congestive heart failure.[148] Excessive α_{2c}-mutant-mediated norepinephrine release at presynaptic nerve endings and the greater β_1-mutant-mediated β_1-AR responsiveness may result in a deleterious state of relative chronic β_1-AR stimulation.

1.3.5. Inflammation stimulates sympathomimetic activity and alters β_1-AR's

The body has many mechanisms that suppress inflammation. Two influential mechanisms are the synthesis of corticosteroids from the adrenal glands and the release of norepinephrine from the sympathetic nervous system. Inflammatory cytokines activate the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system, ultimately elevating both circulating glucocorticoids and catecholamines. [195-199] Both corticosterone and norepinephrine have anti-inflammatory properties[197] and are elevated within 4 days after adjuvant arthritis induction in Lewis rats.[186] Corticosterone levels are significantly elevated by Day 7 in adjuvant arthritic Sprague-Dawley rats.[200]

Norepinephrine exerts its anti-inflammatory affects by interacting with immune cells that express β_2-AR receptors.[201] The resultant suppression of TNF-α, IFN-γ, nitric oxide, and IL-12 release by immune cells helps to limit inflammatory response and tissue damage associated with rheumatoid arthritis.[202] Suppression of the aforementioned cytokines serves to re-balance the T_H1 to T_H2 ratio towards anti-inflammation.[202-204]

Anti-inflammation helps control disease severity, but certain deleterious effects may arise. For example, chronic activation of the sympathetic nervous system predisposes patients to arrhythmia and sudden cardiac death. Essentially, chronic activation of the sympathetic nervous system causes over-stimulation of the β_1-AR. Persistent stimulation of the β_1-AR by norepinephrine reduces β_1-AR sensitivity,[166] and gives rise to homologous desensitization of β_1-ARs by GRKs.[154] The potential for norepinephrine to reduced β_1-AR sensitivity may help to explain the observed reductions in cardiac responsiveness, in humans or rats, to propranolol, sotalol, or verapamil.

1.3.5.1. Norepinephrine

Successful transmission of sympathetic nerve signaling depends on two processes. The first process is for the nerve to elevate local norepinephrine concentrations in the synapse. The second process is signal transduction from the β_1-AR.

The synthesis and release rate of norepinephrine in, and from, the sympathetic nerve is balanced with the removal rate of norepinephrine from the synapse. Tyrosine hydroxylase is the rate limiting enzyme in norepinephrine synthesis. Most of the norepinephrine found in heart tissue is synthesized locally, and therefore norepinephrine is considered a paracrine molecule. Locally, norepinephrine release from peripheral sympathetic nerves is modulated by the inhibitory actions of such receptors as α_2-ARs, and via the stimulatory actions of angiotensin II receptors on the sympathetic nervous system.[205, 206] Stimulation of pre-synaptic α_2-ARs is an example of autoregulation since norepinephrine creates a negative feedback by inhibiting its own release.[202]

In the heart, most norepinephrine is removed by neuronal re-uptake. This is designated as uptake_1.[207] The main component of uptake_1 is the norepinephrine transporter protein, which is a Na^+/Cl^--dependent neurotransmitter symporter located on the plasma membrane.[205] Contributing less than 10% toward circulating norepinephrine under normal conditions,[208] the heart spills less than 1/20th of released norepinephrine transmitter into the venous circulation.[209] During pathogenic states, however, sympathetic nerve outflow may increase to such a degree that up to 500% more norepinephrine is spilled over into plasma.[205, 210]

Reduced norepinephrine transporter density is likely controlled by post-transcriptional modification, possibly by the up-regulation of protein kinase C. Post-transcriptional modification can be instigated in a angiotensin II-dependent or endothelin-dependent manner.[205, 206] Phosphorylation of the norepinephrine transporter results in its internalization.[211] It appears that inflammation reduces norepinephrine transporter density function, even though angiotensin II may upregulate norepinephrine transporter density.[206] The application of nitrogen oxide to PC12 cells (a pheochromocytoma cell line) results in inhibition of norepinephrine uptake.[206] In contrast, however, one ex vivo study demonstrated norepinephrine transporter resistance to oxidative stress inactivation through protective cysteine sites on the norepinephrine transporter.[212] Over all it appears that inflammation reduces norepinephrine transporter functionality, though contradictions in the literature do exist.

A reduction in norepinephrine transporter function reduces norepinephrine uptake through the uptake$_1$ process. At the cellular level, elevated synaptic norepinephrine causes downregulation of β_1-ARs[213] and alterations in the signaling complex.[214] In fact, cardiac interstitial norepinephrine in pacing-induced dog heart failure is inversely correlated with β_1-AR density.[213] It has also been shown that GRK-2, an inactivator of β_1-ARs, is inversely correlated with the density of cardiac norepinephrine transporter,[205, 215] a mechanism that would protect against chronic over-stimulation of the β_1-AR. Administration of norepinephrine to rat cardiomyocytes in vitro decreases β_1-ARs, increases inhibitory G protein alpha subunits, and decreases adenylate cyclase activity.[216] Hence, reductions in norepinephrine transporter density cause the concentration of interstitial norepinephrine to rise, β_1-ARs to be chronically stimulated, β_1-AR density to diminish, and GRK-2 density to elevate.

1.3.6. Cardiovascular risk and sympathetic nervous system activity

Härle et al. 2006 have shown that rheumatoid arthritis causes excessive sympathetic nervous system activity and lower hypothalamic-pituitary-adrenal axis activity: the two systems are out of balance and uncoupled.[185] Via the central nervous system, cytokines activate the sympathetic nervous system. Cytokines also activate the hypothalamic-pituitary-adrenal axis, and ultimately trigger the release of corticosteroids by the adrenal gland.[195] However, in rheumatoid arthritis patients, there is an insufficient response to the rheumatoid-associated inflammation by the hypothalamic-pituitary-adrenal axis, and consequently there is inappropriately low glucocorticoid concentration.[217]

Rheumatoid arthritis variably decreases or increases autonomic nervous system activity.[195] In contrast, sympathetic nervous system activity in rheumatoid arthritis is increased. Juvenile rheumatoid arthritis patients have elevated urine 3-hydroxy-4-phenoxyphenylglycol (a metabolite of norepinephrine), elevated resting heart rate, and reduced cardiovascular response to orthostatic stress tests: all consistent with elevated sympathetic nervous system outflow.[218] Also, cardiac pre-ejection-period is shortened in newly diagnosed rheumatoid arthritis patients;[69] heart rate variability is significantly lower in rheumatoid arthritis patients compared to healthy controls;[219] and there is a relative dominance of the sympathetic nervous system in pupil size control in rheumatoid arthritis patients.[220] Evrengül et al. 2004 studied heart rate variability in rheumatoid arthritis patients and found elevated sympathetic activity and diminished parasympathetic activity.[76] Evrengül et al 2004 suggest alterations in cardiac innervation causes a higher incidence of ventricular tachyarrhythmias, and perhaps explains the presences of excessive cardiovascular death in rheumatoid arthritis patients.[76] This is supported by the fact that ventricular arrhythmia partly depends on β-receptor function.[221] In congestive heart failure patients, β1-AR blockade with metoprolol reduces the rate of sudden cardiac death, probably by reducing fibrillation.[222]

Evidence that sympathetic stimulation in rheumatoid arthritis patients contributes to cardiovascular-related death is provided by comparing Crohn's disease to rheumatoid arthritis. Crohn's disease patients have normal cardiovascular mortality risk,[223] whereas rheumatoid arthritis patients have increased cardiovascular mortality.[68] Whereas rheumatoid arthritis results in a sympathetically driven heart,[69] Crohn's disease patients have a parasympathetically driven heart.[224] A commonality between Crohn's disease and rheumatoid arthritis is elevated circulating inflammatory mediators, including nitric oxide, IL-6, TNF-α, MCP-1, and CRP.[4, 68, 225-230] That is, Crohn's disease patients and rheumatoid arthritis patients have overlapping inflammatory profiles but are in marked contrast with respect to cardiovascular risk. This phenomenon supports the hypothesis that excessive cardiac sympathetic activity increases cardiovascular mortality in rheumatoid arthritis patients.

Like rheumatoid arthritis, congestive heart failure is a disease of excessive myocardial sympathetic activity.[231] Short-term stimulation of the heart improves cardiac output; however, the long-term effects of chronic β_1-AR agonism are deleterious. Chronic β_1-AR agonism increases the probability of developing arrhythmias and reduces myocardial contractility. Potentially, this explains why β-blockers are so beneficial in the treatment of heart failure.[232, 233] Early-stage congestive heart failure is associated with elevated norepinephrine concentration. At first, elevated norephinephrine concentration compensates for reduced cardiac output. However, excessive stimulation of β_1-ARs by norepinephrine gradually becomes detrimental to both heart structure and function.[234] In fact, elevated cardiac norepinephrine concentration is predictive of congestive heart failure-related mortality in human.[135, 137, 235]

Chronic cardiac sympathetic stimulation is associated with abnormal calcium cycling and calcium leaking that reduces contractility and increases arrhymogenicity.[235, 236] Bisoprolol, a β_1-AR blocker, protects rats from developing isoprenaline-induced cardiac fibrosis.[137] This is similar to findings in human, where the use of β-blockers has been widely shown to prevent the progression of congestive heart failure.[237] The beneficial effects of β-blockers may also be related to reduced cardiomyocyte apoptosis and improved Ca^{2+} handling over time.[129, 136] Transgenic mice that over-express β_1-ARs experience progressive cardiac hypertrophy and heart failure. Phospholamban is a protein that inhibits sacroplasmic reticulum uptake of calcium. β_1-AR transgenic mice that are phospholamban knockouts ($PLB^{-/-}$) have greatly increased survival, indicating that altered calcium handling is an important determinant of β_1-AR-related congestive heart failure development.[234] Indeed, the cytotoxic effects of norepinephrine are dependent upon cAMP and calcium influx via verapamil-sensitive channels.[231]

Decreased norepinephrine transporter activity, and density, is associated with heart failure in both animals and in humans. Localized transfection of the norepinephrine transporter into the myocardium of rabbits with congestive heart failure causes structural and functional improvements in pre-existing heart failure.[238] Importantly, the down-regulation of β_1-ARs that occurs with congestive heart failure is reversed by the local transfection of norepinephrine transporter,[238] indicating a strong association between sympathetic drive, norepinephrine concentration, and β_1-AR expression. Reductions in β_1-AR density are reversible in cardiac paced rabbits that have reduced norepinephrine transporter density and norepinephrine uptake.[239] This suggests in congestive heart failure that if the restoration of norepinephrine transporter function occurs, the restoration of β_1-AR function may follow.

1.4. *Anti-inflammation strategies*

In human disease inflammatory processes alter both pharmacokinetics and pharmacodynamics of therapeutic drugs. It is thought that reversing the effects of inflammation would therefore be desirable. Anti-inflammatory therapy is used to decrease the symptoms of rheumatoid arthritis, and to reduce disfigurement and disability. There is an additional role for anti-inflammatory treatment in rheumatoid arthritis. It is for using anti-inflammatory agents with the goal of restoring diminished cardiac pharmacodynamic response.

1.4.1. Non-steroidal anti-inflammatory drugs, disease-modifying anti-rheumatic drugs, spironolactone, and anti-TNF-α drugs

Non-steroidal anti-inflammatory drugs and cyclooxygenase II inhibitors are used for the treatment of pain and stiffness in inflammatory rheumatoid arthritis.[240] Cyclooxygenase II inhibitors have a special place in rheumatoid arthritis treatment, as they cause slightly fewer gastro-intestinal bleeds and ulceration than non-steroidal anti-inflammatory drugs. Both non-steroidal anti-inflammatory drugs and cyclooxygenase II inhibitors are relatively safe for short-term use, but in the long-term even the cyclooxygenase II inhibitors are associated with gastro-intestinal bleeding and ulceration. Ketoprofen, a non-steroidal anti-inflammatory drug, has been shown to reduce arthritic index scores and propranolol AUC in severe adjuvant arthritic rats.[241] However, non-steroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors increase cardiovascular risk in humans.[1, 77] Therefore, the use of either of these agents in the normalization of cardiac pharmacodynamic response may be unjustified.

Early treatment of rheumatoid arthritis with disease-modifying anti-rheumatoid drugs helps prevent disease progression.[242] There are many disease-modifying anti-rheumatoid drugs, including auranofin, azathioprine, cyclosporin, D-penicillamine, hydroxychloroquine, leflunomide, methotrexate, mycophenolate mofetil, sodium aurothiomalate, sulphasalazine, and cyclophosphamide.[242] Hydroxychloroquine, chloroquine, and methotrexate may be useful in improving cardiovascular pharmacodynamic responsiveness because they demonstrate a variety of anti-platelet, anti-thrombogenic, and anti-inflammatory properties.[1]

As discussed in 1.3.5.1, inflammation can decrease the functionality of norepinephrine transporters, and hence β_1-AR function. This is emphasized by three observations: a) IFN-α reduces norepinephrine transporter density and norepinephrine uptake,[243] b) IL-1β stimulates norepinephrine release,[244] and c) nitric oxide reduces norepinephrine uptake.[206]

Spironolactone is a mineralocorticoid receptor antagonist and therefore has diuretic properties which are useful in the treatment of congestive heart failure. Spironolactone has been shown to upregulate norepinephrine transporter density and norepinephrine uptake in salt-sensitive Dahl rats fed high sodium diets.[245] Though not itself an anti-inflammatory agent, spironolactone may facilitate the reversal of inflammatory effects on norepinephrine transporters. This may be an additional reason that spironolactone is beneficial in congestive heart failure treatment. The use of spironolactone, therefore, may also be for reducing cardiac sympathetic nervous stimulation in rheumatoid arthritis patients.

Anti-TNF-α therapy was designed because of TNF-α's pivotal role in the pathophysiology of rheumatoid arthritis. Anti-TNF-α therapeutic agents include infliximab, etanercept, and adalimumab. Anti-TNF-α agents are generally used in patients that have very active disease (two consecutive disease activity scores greater than 5.1), and very persistent disease (having tried at least two disease-modifying anti-rheumatic drugs).[246] Guidelines will vary from country to country. Though infliximab treatment in inflamed rats restores normal QT and PR interval,[125] the anti-TNF-α drugs are very expensive biopharmaceutical agents. Price and safety issues necessarily limit the use of anti-TNF-α drugs for the purpose of restoring cardiovascular pharmacodynamic responsiveness. Similar beneficial effects on improving the pharmacodynamic response of cardiac drugs may exist with anakinra, an anti-IL-1 antibody. Anakinra has proven effectiveness in treating the symptoms of rheumatoid arthritis,[67] however, like the anti-TNF-α drugs anakinra is very expensive and safety concerns exist.

1.4.2. Angiotensin II type 1 receptor blockers

The angiotensin II type 1 receptor blockers have anti-inflammatory properties which supplement their initial therapeutic indications. These drugs are relatively safe, inexpensive, and have widespread use. Potentially, the angiotensin II type 1 receptor blockers could be useful in restoring cardiac pharmacodynamic response in inflamed patients by normalizing receptor signal transduction and therefore function.

Blocking the effects of angiotensin II at the angiotensin II type 1 receptor causes vasodilation, and thereby reduces blood pressure. Reducing blood pressure lowers the stroke risk, myocardial infarction, and congestive heart failure. Clinically, angiotensin blockade may help reduce the complications of diabetes mellitus, and potentially treat the symptoms of asthma or rheumatoid arthritis.[12, 247]

Hanafy et al. 2007 have used the Pre-AA model of inflammation to investigate the effects of valsartan on verapamil pharmacokinetics and pharmacodynamics.[83] Verapamil plasma concentrations were elevated in the Pre-AA group, yet the PR interval responsiveness was reduced. Pre-AA had also reduced L-type calcium channel density and binding capacity in whole cardiac tissue. Valsartan treatment did not normalize verapamil pharmacokinetics. However, twice daily valsartan treatment in Pre-AA animals was shown to significantly reverse: a) the down-regulated PR interval response and b) the L-type calcium channel protein density and ligand-binding capacity. This study demonstrates the potential for angiotensin II type 1 receptor blockers in restoring normal response to verapamil in humans with inflammatory diseases.

1.4.3. Statins

Over 35 years ago cholesterol was shown to be causally linked with coronary heart disease. Soon after this discovery, but still in the 1970's, it was thought that blockade of hydroxymethylglutaryl-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, would prove to be beneficial in hypercholesterolemia and therefore be useful in reducing cardiovascular morbidity and mortality. The long development time for drugs, however, meant that it was not until 1987 when the first statin would be introduced. That drug was lovastatin.[248] Since the 1980's, much more has been learned about the statins as a drug class, including the fact that unexpected anti-inflammatory properties are found across the statin drug class.

1.4.3.1. Pravastatin

The statins are either an open chain or a closed-ring lactone structure. Pravastatin (Figure 1-3) has an open chain structure with a hydroxyl group on the hydrophobic ring structure, giving it the highest water solubility rating of the statins. Pravastatin undergoes less metabolism than the other statins, and it has no active metabolites. Pravastatin also has a very short half-life (1.8 hours). Approximately 50% of pravastatin is recovered unchanged in urine, with much of the rest being metabolized to the 3-hydroxy isomer. While closed-ring lactone statins require metabolism to form the open and active form, pravastatin does not.[248, 249]

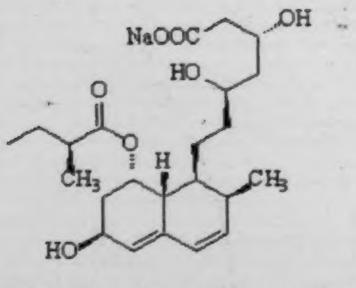

Figure 1-3. Chemical structure of pravastatin[250]

Pravastatin was used a number of large-scale clinical trials to support claims of its effectiveness for reducing cholesterol, and for improving morbidity and mortality. The West of Scotland Coronary Prevention Study (WOSCOPS) was the first cholesterol reduction study to confirm, using pravastatin, that cholesterol reduction lowers cardiovascular mortality without increasing non-cardiovascular mortality rates.[251, 252] Pravastatin treatment lowered low-density lipoprotein levels by 26%, and the resultant relative risk reduction of approximately 30% was independent of age, smoking, plasma lipid profile, or the presence of minor vascular disease.[251]

The Cholesterol and Recurrent Events trial (CARE) confirmed that pravastatin lowers cholesterol by approximately 30%. However, this study also showed that cardiovascular mortality was reduced by 26% even in subjects with normal cholesterol (125-150 mg/dl), which was similar to the reduction (35%) for those with higher cholesterol baselines (150 to 240 mg/dl).[253] In the Long-Term Intervention with Pravastatin in Ischemic Disease study (LIPID), the beneficial effects of pravastatin in patients having widely ranging cholesterol were confirmed. The LIPID study group showed that 40 mg of pravastatin daily reduced all-cause mortality by 22% compared to placebo, and that beneficial effects were seen in all subgroups.[254]

1.4.3.2. Pleiotropic effects of pravastatin

The Cholesterol and Recurrent Events trial demonstrated that patients with comparable lipid levels have different levels of risk of coronary heart disease depending on whether the patient was in the control- or pravastatin-treated group.[255] The pravastatin-treated group had a lower risk of coronary heart disease and myocardial infarct, despite having similar cholesterol levels: a paradox to the normal belief that statins exert their benefits by lowering cholesterol.

Statins reduce the risk of stroke, yet hypercholesterolemia is not a major risk factor for stroke, thereby providing further evidence that alternative mechanisms of benefit exist within the statins. The reduction in the rate of stroke is similar to the reduction in the rate of myocardial infarction.[256] Observations like these indicate that pravastatin has a pleiotropic benefit in cardiovascular risk reduction, as in fact all statins do.

This section details some of the pleiotropic effects of pravastatin that has inspired research in a variety of therapeutic areas including multiple sclerosis, rheumatoid arthritis, nephropathy, diabetes, and bone fracture.[5, 257] Various cellular and mediator effects of pravastatin are listed below in Table 1-4. Essentially, the statins restore endothelial function, enhance atherosclerotic plaque stability, decrease oxidative stress, and reduce inflammation.[255] The one underlying mechanism of statin pleiotropism is decreased isoprenylation of guanosine triphosphate-binding proteins (including Rac and Rho) by farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate. Additionally, pravastatin has antioxidant properties, which is a mevalonate pathway-independent mechanism.[258]

Geranylgeranyl pyrophosphate, an intermediate in the hydroxymethylglutaryl co-enzyme A pathway, is vital in the activation of Rho. Rho proteins normally exist in the geranylgeranylated state,[259, 260] and influence many cellular functions including cellular polarity, endocytosis, cytoskeleton regulation, cell adhesion, differentiation, oncogenesis, NADPH oxidase activity in phagocytes, and gene transcription.[261] Since the statins lower the amount of geranylgeranyl pyrophosphate available for prenylation, the statins therefore also lower the amount of active Rho within the cell membrane.[260, 262] In a study by Bourcier and Todd, 2000, the pleiotropic effects of simvastatin on plasminogen activator inhibitor-1 in human vascular smooth muscle cells and endothelial cells could be reversed by the addition of geranylgeranyl pyrophosphate, which is suggestive of a Rho dependent pathway.[263]

Table 1-4. Pleiotropic effects of pravastatin on inflammation

Parameter	Pleiotropic effect
Markers of inflammation	
CRP	↓ in circulation of humans.[50, 264]
	↓ IL-6-induced CRP production in human hepatocytes.[265]
ICAM-1	↓ in isolated human monocytes.[266]
IFN-γ	↓ in isolated human mixed-lymphocytes.[266]
	↓ in rat Pre-AA.[82]
	↓ in spleen cells of mice incubated with pravastatin.[267]
IL-12	↓ in spleen cells of mice incubated with pravastatin.[267]
	↓ in isolated human mixed-lymphocytes.[266]
IL-1β	↓ in human umbilical vein endothelial cells.[268]
	↓ in circulation of rat post-myocardial infarction.[269]
IL-6	↓ production in human umbilical vein endothelial cells.[268]
	↓ in ex vivo human whole blood.[270]
	↓ in spleen cells of mice incubated with pravastatin.[267]
MCP-1	↓ production in monocytes.[271]
	↓ in spleen cells of mice incubated with pravastatin.[267]
MMP-2	↓ content in human carotid plaque.[272]
MMP-9	↓ activity in primary human monocytes.[273]
TGF-β1	↑ levels in circulation.[274]
T_H1/T_H2 Balance	↓ T_H1/T_H2 ratio in chronic graft-versus-host disease.[275]
TNF-α	↓ production in monocytes.[271]
	↓ production in ex vivo human whole blood.[270]
	↓ in spleen cells of mice incubated with pravastatin.[267]
	↓ in circulation of rat post-myocardial infarction.[269]
Immune cells	
B lymphocytes	↓ B cell proliferation and ↓ humoral rejection in transplantation.[276]
Macrophages	↓ content in monkey atherosclerosis lesions.[277]
T lymphocytes	↓ T cell content of human carotid plaque.[272]
	↓ cytotoxic T lymphocyte activity in human blood.[278]
Transcription Factors	
Nuclear factor-κB	↓ expression in human monocytes.[271]
	↓ activation in lipopolysaccharide-stimulated human monocytes.[279]
PPARα	↑ expression in human monocytes.[271]
	↑ production in hepatocytes and human umbilical vein endothelial cells.[268]

One end-result of statin-dependent Rho inhibition is activation of the anti-inflammatory transcription factor peroxisome proliferator-activated receptors (PPAR)-α.[262] The PPARs are a set of nuclear receptors. PPAR-α, γ, and β/δ, have effects on adipocyte differentiation, insulin sensitivity, and fatty acid storage and oxidation.[280-282] PPAR-γ's primary role is that of regulation of energy and glucose metabolism.[283]

Nuclear factor-κB is a pro-inflammatory transcription factor responsible for the production of mediators that induce local and systemic inflammation.[284] In the inactive state, nuclear factor-κB remains in the cytoplasm though association with the inhibitor of κBs (IκBs). Activation of nuclear factor-κB occurs when IκB becomes phosphorylated by the inhibitor of IκB kinase (IKK), after which time IκB dissociates from nuclear factor-κB. Nuclear factor-κB then enters the nucleus where it interacts with DNA and acts as a transcription factor. In the nucleous, nuclear factor-κB becomes de-activated by association with its inhibitor, IκB, at which time the complex is relocated back out into the nucleoplasm.[284]

The nuclear factor-κB pathway becomes activated though various factors including reactive oxygen species, viral and bacterial infections, and the presence of pro-inflammatory cytokines. This is a major pathway for the production of many inflammatory mediators including, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, macrophage-chemotactic protein-1, TNF-α, IL-1β, IL-2, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and the MMPs. The nuclear factor-κB pathway also initiates production of inflammatory enzymes including inducible nitric oxide synthase, cyclooxygenase-2, and phospholipase-A2, and acute-phase proteins such as angiotensinogen and serum amyloid A precursor.[284-287] It is also involved in the production of IFN-γ, which is required for cellular-mediated T_H1 responses.[288]

Excessive inflammation in the cardiovascular system is detrimental. The Cholesterol And Recurrent Events study (CARE) confirmed that vascular risk is associated with high concentrations of CRP, and that pravastatin lowers inflammation, and also lowers vascular risk.[33, 289] CRP concentrations are a strong independent predictor of risk of myocardial infarct and stroke in apparently healthy men and women, and the Pravastatin Inflammation/CRP Evaluation trial (PRINCE) showed that statins reduce CRP in a manner independent of cholesterol reduction.[264] In monkeys, pravastatin increases plaque stability by improving endothelial function and by reducing lesional macrophage content, calcification, and neovascularization.[33] In the LIPID study, white blood cell counts were shown to be predictive of coronary heart disease mortality, and those patients with highest white blood cell counts responded best to pravastatin.[290] It had also been shown that pravastatin reduces thrombin formation.[291] These facts together, have shown how pravastatin reduces cardiovascular risk independently of cholesterol reduction, via anti-inflammatory mechanisms.

Nervous system activity in the heart is an important component of cardiovascular disease. However, excessive sympathetic activity is associated with arrhythmia and sudden cardiac death. In contrast, parasympathetic activity is protective against arrhythmia. Eight weeks of pravastatin, but not simvastatin treatment, increases the cardiac response to parasympathetic activity in patients at high risk of a cardiovascular event. How pravastatin influences sympathetic activity is unknown, however, atorvastatin treatment does appear to reduce sympathetic activity in heart failure patients.[292]

1.4.3.3. Pleiotropic effects of other statins which influence cardiovascular function or rheumatoid arthritis

Although studies have shown conflicting results for the use of statins in the treatment of experimental arthritis,[293, 294] human data is better. In a small study of 8 rheumatoid arthritis patients, simvastatin has been shown to significantly reduce patient self-assessment of disease activity by 13% ($p=0.03$).[295] In a recent and larger study of 140 patients, atorvastatin reduced disease activity scores by 0.5 (possible scores range from 0 – 10) over the 6 month study, whereas placebo patients only had an increase of 0.03 ($p=0.004$).[296] Importantly, atorvastatin also significantly reduced several markers of vascular risk including CRP ($p<0.0001$), erythrocyte sedimentation rate ($p=0.005$), and IL-6 ($p=0.028$).[296]

The use of simvastatin (20 mg daily) for 8 weeks in long-term rheumatoid arthritis patients improves endothelium-dependent vascular function, and reduces the concentration of TNF-α (p=0.012) and CRP (p=0.025).[297] Others have shown that simvastatin improves endothelium-dependent vascular function resulting in higher flow-mediated vasodilatation compared to placebo (p=0.02).[298] Stratified by low vs. high CRP, high CRP patients treated with simvastatin show a significant increase in flow-mediated dilatation (p=0.04). These data suggest that statin use could reduce cardiovascular risk in rheumatoid arthritis patients.

Snow and Mikuls 2005 suggest that the anti-inflammatory effects of statins will help reduce excess cardiovascular mortality in rheumatoid arthritis patients.[60] Perhaps the depressor effects of statins on the sympathetic nervous system will also help reduce mortality. Simvastatin normalizes renal sympathetic nerve outflow in an experimental pacing-induced chronic heart failure in rabbits.[299] This model of congestive heart failure is associated with an abnormal baroreflex regulation that is normalized by 66% when treated with simvastatin. The combination of anti-inflammatory properties that improve vascular function and anti-sympathetic effects that may help reduce arrhythmia predisposition show promise for statins in preventing cardiovascular risk in rheumatoid arthritis patients.

1.4.3.4. Clinical studies on the pleiotropic effects of pravastatin

Due to beneficial effects, the statins as a group are being investigated as therapeutic agents in a wide variety of diseases. This section will outline some of the major clinical studies that have investigated the pleiotropic effects of pravastatin; however, cholesterol reduction-related studies will not be covered.

One of the first studies to test the pleiotropic effects of statins was conducted in 1994 by Egashira et al. 1994. In this 6 month study, pravastatin was administered to see if it could improve coronary blood flow and microcirculation.[300] The outcome of the study was that pravastatin increased myocardial perfusion, but that it was not independent of low-density lipoprotein levels. Also, the effects were limited to hypercholesterolemic patients, and were not observed in control subjects.[301] Other studies have shown pleiotropic effects that are independent of cholesterol. Table 1-5 briefly describes the outcomes of studies that have investigated the pleiotropic effects of pravastatin administration in humans.

Table 1-5. Studies investigating the pleiotropic (non-cholesterol lowering) effects of pravastatin in human.

Trial	**Pleiotropic effect with pravastatin administration**	**Outcome**
Borghi et al. 2000[302]	Hypertension	Positive; blood pressure lowering independent of lipid effect.
CARE[289]	Inflammation reduction in risk of non-fatal myocardial infarct or fatal coronary event is associated with reductions in CRP and serum amyloid A *	Positive; inflammation (post-myocardial infarct) is associated with risk that can be attenuated by pravastatin administration.
Crisby et al. 2001[272]	Plaque stabilization; carotid artery plaques	Positive; reductions in lipids, lipid oxidation, inflammation, MMP-2, and cell death. Increases in TIMP-1 and collagen.
Egashira et al. 1994[301]	Endothelial function	Positive; improves myocardial perfusion, but not independent of low-density lipoprotein.
Horwich et al. 2004[303]	Heart failure	Positive; improves survival independently.
Jialal et al. 2001[304]	Inflammation; reduction of CRP	Positive; reductions in CRP independent of low-density lipoprotein.
Kobashigawa et al. 1995[305]	Cardiac transplantation; improvement of outcome	Positive; lower rejection rates. Independence of cholesterol only suggested, not proven.
Lee et al. 2002[306]	Left ventricular mass	Positive; reduced left ventricle mass independent of lipids.
LIPID[307]	Bone fracture *	Negative; no changes.
Nishikawa et al. 2002[308]	Coronary artery disease	Positive; treatment developed coronary collateral circulation.
PREVEND-IT[309]	Urinary albumin excretion and glomerular filtration rate	Negative; no changes.
PRINCE[284]	Inflammation; reduction of CRP	Positive; reductions in CRP independent of low-density lipoprotein.
PROSPER[310]	Cerebral blood flow or parenchymal volume *	Negative; no changes.
Wolozin et al. 2000[311]	Alzheimer's disease (cross-sectional study)	Positive; 60-73% lower prevalence in people taking pravastatin or lovastatin.
WOSCOPS[312]	Diabetes mellitus *	Positive; 30% reduction in probability of diabetes onset.

*secondary analysis of a study having a different primary endpoint.

Currently there are many on-going clinical trials that are investigating the pleiotropic effects of pravastatin. According to the United States National Institutes of Health Clinical Trials database these studies include the: 1) amelioration of renal and cardiovascular disease in autosomal dominant polycystic kidney disease; 2) prevention of recurrent stroke; 3) treatment of small cell lung cancer; 4) treatment of relapsing-remitting multiple sclerosis; and 5) improvement of residual symptoms and cognitive function in schizophrenia.[313] Perhaps the number of potential uses for pravastatin is so large due to its interference in such a critical biochemical pathway. Perhaps there will be more research into the use of pravastatin as a therapeutic agent in rheumatoid arthritis, or even as a preventive agent for cardiovascular events associated with rheumatoid arthritis.

1.5. *Rationale for this thesis*

- Patients with rheumatoid arthritis have excessive cardiovascular morbidity and mortality. Cardiovascular treatment in these patients is obviously intended to reduce risk. However, treatment failure is a risk due to the presence of inflammation.
- Cardiovascular response to propranolol is diminished in animal models of rheumatoid arthritis, even in the presence of elevated plasma concentrations of propranolol. Also, cardiovascular response to verapamil is reduced in humans with rheumatoid arthritis, as is propranolol response in other animal models. These facts may facilitate increases in the morbidity and mortality of rheumatoid arthritis patients.
- Pravastatin has many anti-inflammatory properties. Consequently, pravastatin may reduce elevated propranolol concentration and diminished cardiovascular response in the Pre-AA model of inflammation. The anti-inflammatory effects of pravastatin may therefore offer therapeutic potential for rheumatoid arthritis patients

who have co-morbid cardiovascular disease, and hence minimize cardiovascular-related morbidity and mortality.

1.6. *Hypotheses*

A. In the rat, the Pre-AA, model of inflammation,
    a. as defined by 8 days post-complete Freund's adjuvant injection, causes a T_H1-skewed inflammatory response;
    b. increases circulating propranolol concentration;
    c. attenuates PR interval prolongation (dromotropic response) during propranolol administration.

B. In Pre-AA rats, the anti-inflammatory effects of pravastatin,
    a. normalize circulating markers of inflammation;
    b. reduce (normalize) circulating propranolol concentration;
    c. restore propranolol response as measured by PR interval.

C. From a mechanistic perspective, pravastatin restores propranolol response in Pre-AA rats by,
    a. altering the sympathetic nervous system;
    b. influencing matrix metalloproteinase activity;
    c. normalizing the β_1-adrenergic receptor signaling pathway.

1.7. *Objectives*

A. Pharmacodynamics
    a. Using PR interval analysis, to assess cardiovascular response to propranolol in Pre-AA rats.
    b. Using PR interval analysis, to determine the effect of pravastatin on cardiovascular response to propranolol in Pre-AA animals.

B. Pharmacokinetics
    a. To determine the effects of Pre-AA on propranolol exposure after oral dosing.
    b. To determine if pravastatin normalizes propranolol pharmacokinetics in Pre-AA.

C. Inflammatory profile of Pre-AA and the effects of pravastatin
    a. To characterize the inflammatory profile of Pre-AA using: nitric oxide metabolites, CRP, IFN-γ, IL-10, MMP-2, and MMP-9.
    b. To determine how pravastatin alters the inflammatory profile of Pre-AA.

D. β1-adrenergic receptor signaling changes
    a. To characterize alterations in sympathetic signaling that are associated with Pre-AA and pravastatin administration which ultimately influence β1-adrenergic receptors. This will be achieved by assessing norepinephrine concentration and norepinephrine transporter density.
    b. During Pre-AA and pravastatin administration, to assess critical proteins involved in β_1-AR signal transduction, including the β_1-AR, G-protein-coupled receptor kinase-2, and L-type calcium channels.

2. Pravastatin Reverses the Down-Regulating Effect of Inflammation on β-Adrenergic Receptors: A disease-drug interaction between inflammation, pravastatin, and propranolol.[A]

2.1. *Introduction*

Inflammatory conditions reduce cardiac response to β-adrenergic antagonists such as propranolol[114] and sotalol[124] in rats, and to the calcium channel antagonist verapamil in humans.[4] This reduced response can occur despite the fact that inflammation elevates propranolol and verapamil concentrations.[4, 114]

Rheumatoid arthritis is associated with excessive cardiovascular morbidity and mortality, and is accompanied by elevated markers of inflammation. Statins have anti-inflammatory properties and reduce cardiovascular-related death.[50]

Pre-AA is a rat model of rheumatoid arthritis that is produced by injection of complete Freund's adjuvant which results in elevation of pro-inflammatory mediators but is not associated with pain and discomfort.[79]

2.2. *Hypotheses*

A. Pre-AA
   a. Pre-AA causes a T_H1-skewed inflammatory response.
   b. Inflammation associated with Pre-AA significantly increases circulating propranolol concentration.
   c. Propranolol response in Pre-AA attenuates PR interval (dromotropic response).

[A] A version of this chapter has been published. Clements JD, Jamali F. Vascular Pharmacology 2007;46:52-59.

B. Pravastatin

The anti-inflammatory effects of pravastatin, in Pre-AA animals:

- a. normalizes circulating markers of inflammation.
- b. reduces (normalize) circulating propranolol concentration.
- c. restores propranolol response as measured by PR interval.

2.3. *Objectives*

A. Pharmacodynamics

- a. To assess cardiovascular response to propranolol in Pre-AA rats using PR interval analysis.
- b. To determine the effect of pravastatin on cardiovascular response to propranolol in Pre-AA rats using PR interval analysis.

B. Pharmacokinetics

- a. To determine if Pre-AA causes changes in propranolol exposure after oral dosing.
- b. To determine if pravastatin normalizes propranolol pharmacokinetics in Pre-AA.

C. Inflammatory profile of Pre-AA and the effects of pravastatin

- a. To characterize the inflammatory profile of Pre-AA by assessing the concentration of nitric oxide, CRP, IFN-γ, and IL-10.
- b. To determine how pravastatin alters the inflammatory profile of Pre-AA.

2.4. *Methods*

2.4.1. Materials

Racemic propranolol HCL, Aspergillus nitrate reductase (10 U ml-1), HEPES, flavin adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, lactate dehydrogenase (1500 U ml-1), pyruvic acid, naphthyleneethylenediamine dihydrochloride, sulfanilamide, sodium nitrite, and sodium nitrate were purchased from Sigma Chemical Co. (St. Louis, MO).

Phosphoric acid and sodium hydroxide were purchased from BDH Chemicals (Toronto, Canada). Killed, desiccated Mycobacterium butyricum was purchased from Difco Laboratories (Detroit, MI). Pravastatin sodium tablets were acquired from Apotex (Toronto, Canada). High-performance liquid chromatography (HPLC) grade hexane, chloroform and methanol were purchased from Caledon Laboratories (Georgetown, Canada). Rat IFN-γ and IL-10 ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN). Rat CRP ELISA kit was purchased from Helica Biosystems Inc. (Fullerton, CA). Teflon-coated wire used for the electrocardiograph electrodes was purchased from Biomed Wire (Chatsworth, CA). Jugular vein catheters were constructed from PE-50 polyethylene purchased from Clay Adams (Parsippany, NY) and silastic tubing purchased from Dow Corning Corporation (Midland, MI). The MPS Micropartition Device used in the protein binding experiment was purchased from Millipore (Billerica, MA).

2.4.2. Animals

The study was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. Adult male Sprague-Dawley rats were used in pharmacodynamics (245±22 g, n=14-16/group) and pharmacokinetics (280 ± 30 g, n=5-9/group) experiments. The animals were housed in a 12 h light/dark cycle and always had free access to water, though food was withheld for 12 hours before propranolol dosing and 3 hours afterwards. A halothane/O_2 mixture was used to achieve deep anesthesia before any invasive procedures.

2.4.3. Pre-AA

To induce Pre-AA, 38 mg/kg of triturated desiccated *Mycobacterium butyricum* (squalene suspension, 50 mg/ml) was injected into the base of the tail under anesthesia; placebo consisted of 0.9% normal saline. The experiment was conducted over 8 days post adjuvant injection and before the emergence of physical signs of adjuvant arthritis such as swollen paws and inflamed joints.

2.4.4. Propranolol Pharmacodynamics

Rats were randomly allocated into four groups: Healthy/Placebo (n=14), Healthy/statin (n=16), Arthritis/Placebo (n=15), and Arthritis/statin (n=16). The rats were anesthetized and Teflon-coated electrocardiograph electrodes were implanted subcutaneously into the left and right axial regions. On the next day (corresponding to Day 0) the baseline (pre-propranolol) PR and RR interval was recorded using Acknowledge 3.01 data acquisition system (BIOPAC Systems Inc. Santa Barbara, CA). Subsequently, oral racemic propranolol 25 mg/kg (in 1% methylcellulose/water) was administered by oral gavage and PR and RR intervals recorded at 20, 40, 60, 80, 100, 140, 180, and 240 min. Electrocardiogram response, based on 6 consecutive cycles, to propranolol was recorded on Day 0, and then on Day 4 and 8 days post adjuvant injection. To test the effect of pravastatin on propranolol potency to prolong PR interval, the Healthy/Pravastatin and Pre-AA/Pravastatin group animals received twice-daily 6 mg/kg oral pravastatin from Day 4 to Day 8 and the Healthy/Placebo and Pre-AA/Placebo group received placebo (vehicle) from Day 4 to Day 8. After electrocardiographic recordings on Days 4 and 8, rats were anesthetized and approximately 0.3 ml blood was collected from the tail vein for inflammation marker measurement. On Day 4, only plasma nitrite concentrations were measured. On Day 8 plasma nitrite, serum CRP, IFN-γ and IL10 were measured were measured 7 hours after the last dose of pravastatin. Samples were stored at -80° until analyzed.

Measurement of PR and RR-intervals was performed under blinded conditions so that the investigator was unaware of the identity of the data set. From the start of the P-wave to the beginning of QRS complex was considered as the PR interval. At each recording time, six consecutive heart beats were collected and entered into statistical assessment. RR was a full cycle. Percent changes from baseline effect were calculated and plotted vs. time. The area under the % change-time (AUEC, %.min) was calculated using the trapezoidal rule. Percent maximum effect was the highest observed value.

Preliminary experiments related to choosing propranolol as a PR interval probe are described in Chapter 5 (Appendix of miscellaneous experiments).

2.4.5. Propranolol Pharmacokinetics

Rats were randomly allocated into four groups: Healthy/Placebo (n=5), Healthy/Pravastatin (n=5), Arthritis/Placebo (n=9), and Arthritis/Pravastatin (n=9). As described under "Propranolol Pharmacodynamics", rats received *Mycobacterium butyricum* or placebo on Day 0. During the Day 4 to Day 8 period, they received twice-daily 6 mg/kg oral pravastatin or placebo. On Day 7, they were anesthetized and fitted right side with a jugular vein polyethylene catheter tipped with silastic tubing that was externalized dorsally in the interscapular region. On Day 8 the rats received 25 mg/kg of oral racemic propranolol after a 12 h fasting. Serial blood samples (300 µl) were collected at 0, 30, 60, 120, 180, and 240 min post propranolol dosing. Catheters were flushed with 10 IU/ml heparinized saline after sampling. The plasma was separated by centrifugation then stored at -80° until they were analyzed for propranolol. Plasma propranolol enantiomer concentrations were plotted vs. time and the area under plasma concentration-time curve (AUC) was measured using the trapezoidal rule. No other pharmacokinetic parameter was measured.

2.4.6. Propranolol Protein Binding

The extent of propranolol protein binding for the Arthritis/Placebo and Arthritis/Statin animals was determined using a commercially available micropartition device. For each of the two Pre-AA groups, 9 plasma samples were divided in 3 groups each containing 3 samples. To assess R- and S-propranolol unbound fraction we added to a separate group of samples, racemic propranolol to achieve 700 and 7000 ng/ml of S and R enantiomers, respectively. Spiked plasma was incubated at 37° for 1 h, transferred into the micropartition device, and centrifuged at 900 g at 24° for 1 h. To account for non-specific binding, pre-filtered plasma was spiked with propranolol then was subjected to the same conditions as the pooled plasma. The filtrate was then assayed for propranolol using HPLC.

2.4.7. Propranolol HPLC Assay

A previously published stereospecific assay was used to assess rat plasma propranolol enantiomer concentrations.[113] To 100 µl of plasma, 25 µl of internal standard (bupranolol, 10 µg/ml in distilled H_2O) and 250 µl of 0.2 M NaOH were added. The mixture was vortexed for 30 seconds using 5 ml of diethyl ether. Test tubes were dipped into acetone in dry ice and the organic layer was poured into clean test tubes. The organic layer was evaporated to dryness using a Savant Speed Vac concentrator-evaporator (Emerston Instruments, Scarborough, Canada). The residue was derivatized with 185 µl of 0.02% (S)-(+)-1-(1-Naphthyl)-ethyl isocyanate (Sigma-Aldrich, USA) in 50:50 hexane: chloroform followed by vortex-mixing for 60 seconds. The diastereomeric samples were separated using HPLC. The mobile phase, hexane: chloroform: methanol 75:25:0.45, was pumped at 2 ml/min through a 25 cm X 4.6 mm stainless steel Partisil 5 (Phenomenex) silica column. The HPLC system consisted of Waters 501 pump (Millipore-Waters, Mississauga, Canada), a Shimadzu SIL 9A auto injector (Shimadzu, Japan), and a Shimadzu CR601 Chromatopac integrator (Shimadzu, Japan). The detector was a Waters 474 scanning fluorescence detector (Millipore-Waters, Mississauga, Canada) and was set at 280 nm for excitation and 340 nm for emission. Calibration curves were linear for the propranolol enantiomers over the 12.5 – 5000 ng/ml range (r^2>0.99). The minimum quantifiable concentration was set at 12.5 ng/ml (CV<20%).

2.4.8. Assay of Inflammation Biomarkers

The biomarkers were assayed in the serum or plasma harvested from blood samples collected via the tail vein.

For IFN-γ (n=14-16) an enzyme-linked immunosorbent assay was used. 50 µl of serum was transferred to an anti-rat-IFN-γ coated 96 well plate, followed by 2 hours incubation and 5 washings with the kit's wash buffer. Horseradish peroxidase-conjugated anti-rat-IFN-γ was added to the wells followed by 2 hours incubation then 5 washings. Substrate solution containing stabilized hydrogen peroxide and stabilized chromogen (tetramethylbenzidine) was added to each well, followed by 30 min incubation at room temperature. The reaction was stopped with diluted HCl then read at 450 nm with a 543 nm correction. The standard curve was linear over the range of 31.2 to 2000 pg/ml (r^2=0.997) and the lower detectable limit was set at 10 pg/ml, while the lower limit of quantification was 31.2 pg/ml.

IL-10 (n=14-16) was also assayed using an enzyme-linked immunosorbent assay. 50 µl of serum was transferred to an anti-rat-IL-10 coated 96 well plate, followed by 2 hours incubation and 5 washings. Horseradish peroxidase-conjugated anti-rat-IL-10 was then added to the wells followed by 2 hours incubation then 5 washings. Substrate solution containing stabilized hydrogen peroxide and stabilized chromogen (tetramethylbenzidine) was added to each well, followed by a 30 min incubation period at room temperature. The reaction was stopped with diluted HCl then read at 450 nm with a 543 nm correction. The standard curve was linear over the range of 31.2 to 2000 pg/ml (r^2=0.988) and the lower detectable limit was 10 pg/ml.

As a marker of plasma nitric oxide (n=14-16) we assessed the concentration of its stable breakdown products, nitrite (NO_2^-) and nitrate (NO_3^-) using a previously described Griess reaction.[314] Collectively, nitric oxide, NO_2^-, and NO_3^- are referred to as NO_X^-. First, 10 µl Aspergillus nitrate reductase (10 U/ml) was added to 100 µl of plasma, and incubated for 30 min at 37°C in the presence of 25 µl of 1 M HEPES buffer (pH 7.4), 25 µl of 0.1 mM FAD, 50 µl of 1mM NADPH. Then 5 µl of 1500 U/ml lactate dehydrogenase and 50 µl of 100nM pyruvic acid was added and incubated for an additional 10 min at 37°C. Total nitrite concentration was quantified by adding 1 ml of Griess reagent and incubating for 10 min at room temperature for 10 min before an absorbance scan at 543 nm. Calibration curves were used for both nitrite and nitrate, confirming a nitrate reductase efficiency of >99%. The standard curve was linear over the range of 12.5 to 200 µM (r^2>0.99), and the lower limit of quantification was 12.5 µM (CV<15%).

CRP (n=4-6) was assayed using an enzyme-linked immunosorbent assay. 100 µl of a 1:10,000 dilution of serum samples was added to an anti-rat-CRP coated 96 well plate, followed by 30 min incubation at room temperature then 5 washings. Horseradish peroxidase-conjugated anti-rat-CRP was added to the wells followed by 30 min incubation and 5 washings. Substrate solution containing urea peroxide and chromogen (tetramethylbenzidine) was added to each well, followed by 8 min incubation. The reaction was stopped with diluted phosphoric acid then read at 450 nm. The standard curve was linear over the range of 17.5 to 133 µg/ml (r^2=0.988), and the lower detectable limit of the assay was 2.5 µg/ml.

2.4.9. Data Analysis and Statistical Assessment

Pharmacodynamic responses were tested for statistical differences using Statistical Analysis Systems (SAS) software version 8.2 (SAS Institute, USA). The MIXED procedure repeated-measures ANOVA with sub-sampling and a Tukey's adjustment for multiple comparisons was performed to test the significance of the differences in PR interval throughout the 0-240 min experiment time. Significant differences amongst the main treatment effects were reported. Data are presented as both the percent change from baseline for individual mean observations as well as the mean AUEC values. Pharmacokinetic data was evaluated for homogeneity of variances using an F-test then compared using a two-tailed T-Test. Differences in maximum effect, IL-10, NO_X^-, and CRP data were all tested for significance using the general linear model (GLM) procedure with a Tukey's adjustment for multiple comparisons. Since there was a preponderance of data falling below the minimum quantifiable limit in the IFN-γ data, and no rats in either of the healthy groups demonstrated quantifiable IFN-γ, a categorical data modeling (CATMOD) procedure was used to test for a significant difference between the two Pre-AA groups. Data is presented as the mean±SEM, and p-values less than 0.05 were considered significant for all statistical tests.

2.5. *Results*

2.5.1. Development of Pre-AA

By Day 4, adjuvant significantly elevated plasma nitrite concentrations, which was the only inflammatory mediator assessed on this day (Arthritis 110.5±13 vs. Healthy 49.5±4 µM, $p=0.001$). On Day 8, in addition to the elevated nitrite, IFN-γ concentration was significantly greater in the Pre-AA and compared to the Healthy groups (Figure 2-1). Indeed, IFN-γ concentrations were below the assay's quantifiable limit for all Healthy rats. There were no significant differences in serum concentrations of IL-10 and CRP between any groups (Figure 2-1). As expected, no physical AA symptoms, such as paw swelling, were apparent during the study days.

Pravastatin treatment significantly lowered the elevated concentration of IFN-γ (Figure 2-1A) but had no significant effect on other mediator concentrations (Figure 2-1). In the healthy rats, pravastatin did not alter any pro-inflammatory mediator concentrations (Figure 2-1).

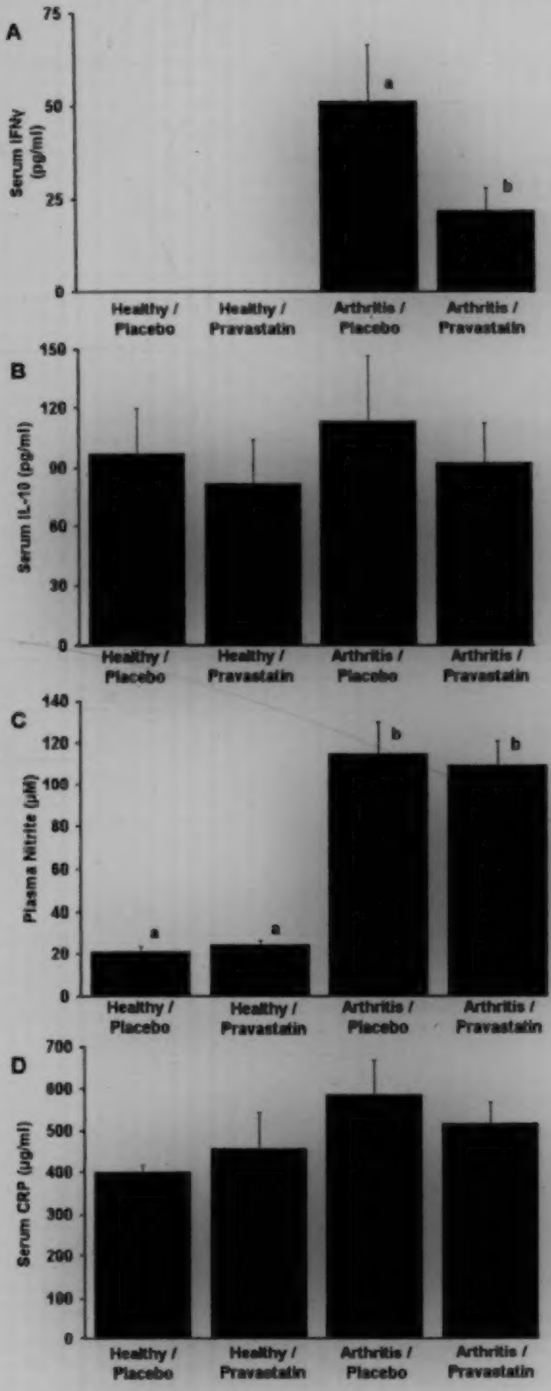

Figure 2-1. The effect of inflammation on inflammatory mediators in the presence and absence of 4 days of pravastatin treatment. The data represent observations 8 days after injection of the adjuvant. Error bars represent standard error of the mean. Keys: IFN-γ, IFN-γ; IL-10, IL-10; CRP, CRP. Healthy groups had no IFN-γ above the quantifiable limit. Same character (a, b) indicates the same statistical grouping. (Fig. 2a – c n=14-16, and Fig. 2d n=4-6).

2.5.2. Propranolol Pharmacodynamics and Pharmacokinetics

Propranolol administration on Days 0 and 4 resulted in a significant PR interval prolongation at all measured time points ($p<0.01$). There were no significant differences in any of the parameters amongst any of the groups on either Day 0 or 4. The mean AUEC (%.min) ranged from 756±262 to 1410±160 for Day 0 and from 742±239 to 1380±280 for Day 4 amongst the four groups.

On Day 8 (Figure 2-2), we showed that healthy groups experienced a significant prolongation of PR interval, whereas inflammation resulted in a significantly reduced propranolol response as compared with the healthy condition. Pravastatin administration restored the reduced propranolol response. In fact, a comparison of Day 8 PR interval recordings with their matching Day 0 values revealed that only the Arthritis/Placebo group demonstrated diminished response to propranolol ($p=0.001$) (Figure 2-3).

As expected,[114] inflammation dramatically increased both propranolol enantiomer plasma concentrations (Figure 2-4, Table 2-1). The AUC was greater by 7-15 fold for both enantiomers in the Arthritis groups as compared to their corresponding Healthy groups (Table 2-1).

On Day 8, after four days of treatment with pravastatin, both healthy and inflamed rats demonstrated an increase in propranolol response (Figure 2-2 and Figure 2-3). The maximum propranolol effect, which occurred at 88±62 -125±67 minutes post-dose in all groups, was 7.7±4.8 % in Healthy/Placebo; 6.0±4.5 % in Arthritis/Placebo; 12.8±8.1 % in Healthy/Statin, and 12.8±7.9 % in Arthritis/Statin. Here, pravastatin administration to either healthy or inflamed animals resulted in a significantly higher maximum PR interval than the Arthritis/Placebo group ($p=0.030$, and $p=0.031$ respectively).

RR interval (heart rate) was prolonged by propranolol by 3 to 15% in all groups and as expected [114], and there were no significant differences between groups. Pravastatin also did not influence propranolol effect on RR.

Pravastatin treatment did not influence propranolol pharmacokinetics in healthy animals. Neither did it normalize the observed reduced clearance secondary to inflammation (Figure 2-4, Table 2-1). In addition, pravastatin did not significantly influence the unbound fraction of propranolol in inflamed rats' plasma (Table 2-1), and therefore differences in free fraction can not explain the differences in propranolol response.

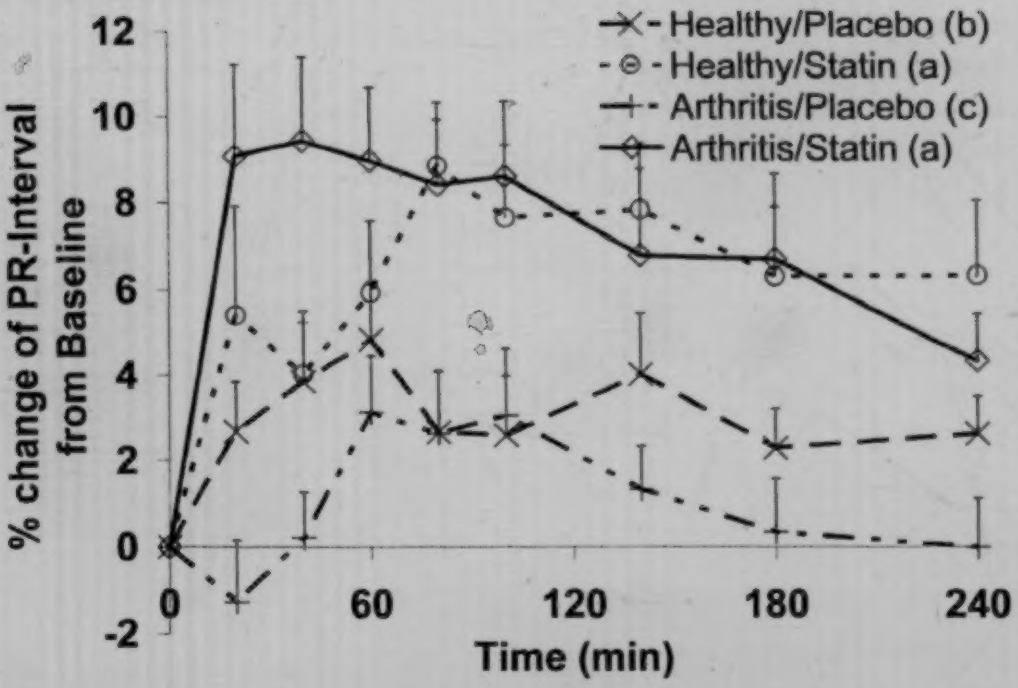

Figure 2-2. Percent change from baseline in PR prolongation following the administration of propranolol to healthy and inflamed (Arthritis) rats in the presence and absence of 4 days of treatment with pravastatin. The data represent observations 8 days after injection of the adjuvant. Same character (a, b, c) indicates the same statistical grouping. (n = 14-16/group).

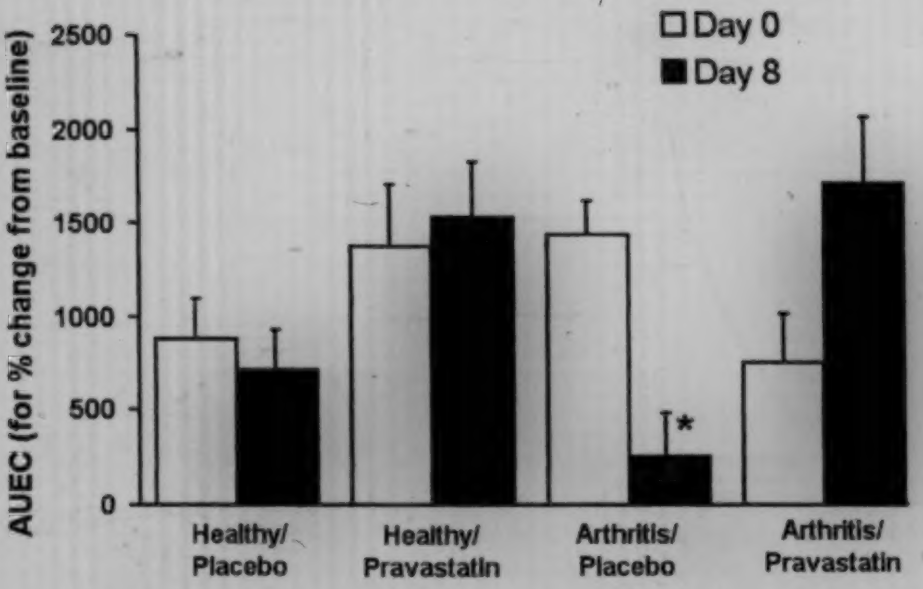

Figure 2-3. The area under the effect curve (% prolongation of PR interval from baseline) following administration of propranolol. Open bars – Day 0 baseline response, before the induction of inflammation or the administration of pravastatin. Solid bars – Day 8 response in healthy or inflamed rats, with or without 4 days of pravastatin treatment. Compared to their own Day 0 baseline values, only the Arthritis/Placebo group showed reduced propranolol response ($p<0.001$), whereas the Arthritis/Pravastatin group showed a non-significant trend for increased propranolol response ($p=0.051$). Comparison of Day 8 data showed that the Arthritis/Placebo group had the lowest response.

*Significantly different from Day 0. (n = 14-16/group).

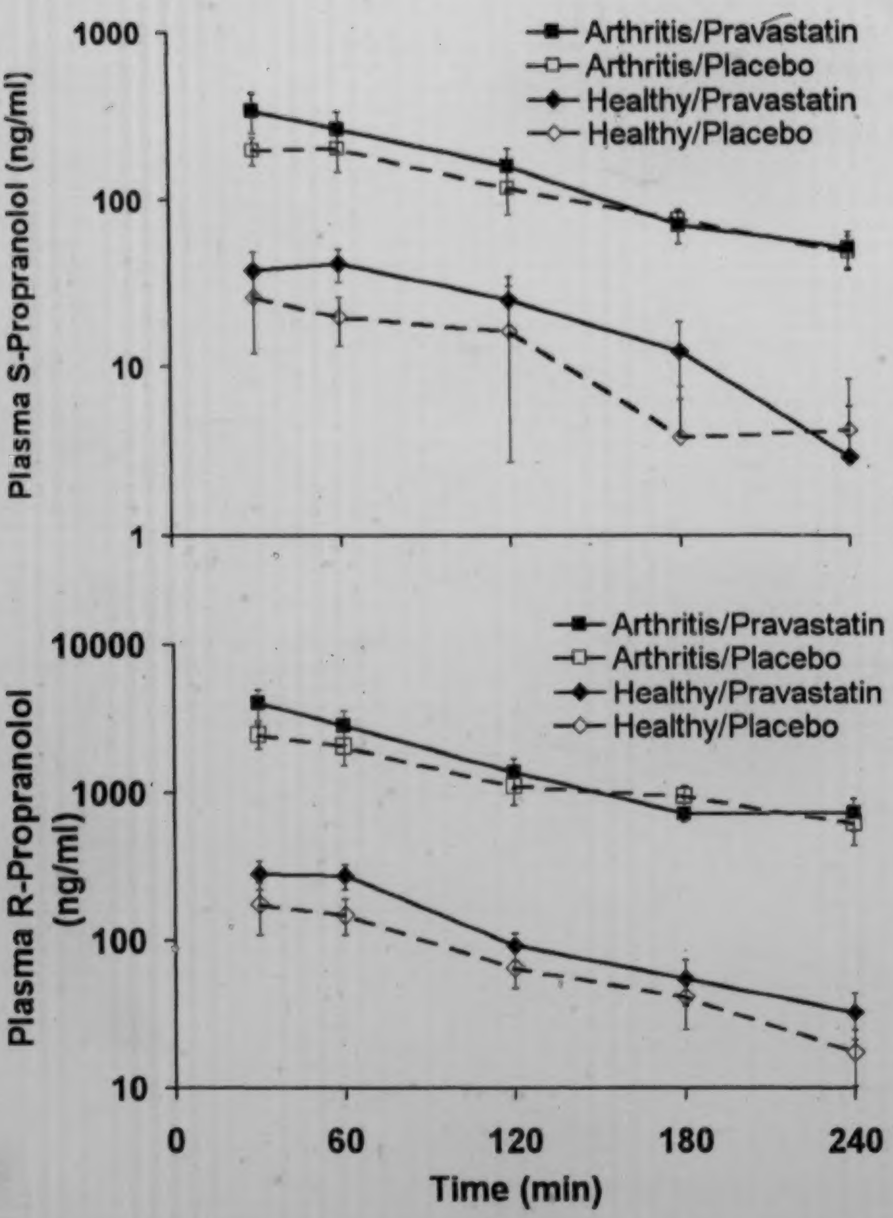

Figure 2-4. Plasma concentration-time course of S- and R-propranolol following administration of racemic propranolol to healthy (n=5/group) and inflamed (Arthritis, n=9/group) rats in the presence and absence of 4 days of pravastatin treatment (8 days after injection of the adjuvant to the inflamed groups).

Table 2-1. Pharmacokinetic indices (mean±SEM) of S- and R-propranolol following oral administration of 25 mg/kg of racemic propranolol to healthy (n=5/group) and inflamed (Arthritis, n=9/group) rats in the presence and absence of 4 days of pravastatin (Statin) treatment (8 days after injection of the adjuvant to the inflamed groups).

		Placebo		Statin	
		R	S	R	S
Healthy:	$\mathrm{AUC}_{0\text{-}4h}$ (µg.min/ml)	19 ± 13	3.0 ± 4	31 ± 12	5.3 ± 3
Arthritis:	$\mathrm{AUC}_{0\text{-}4h}$ (µg.min/ml)	310 ± 182[*]	28 ± 19[*]	396 ± 245[*]	38 ± 27[*]
	% Unbound[†]	22 ± 3	2 ± 1	15 ± 8	2 ± 2

[*] Significantly different from corresponding Healthy groups.

[†] Fraction unbound was measured for the arthritis group only.

2.6. *Discussion*

The present data suggest, for the first time, that the diminishing effect of inflammation on β-adrenergic response appears to be reversed by pravastatin. We have confirmed the observations of Guirguis and Jamali (2003), showing that inflammation significantly increases plasma propranolol concentration (Figure 2-4, Table 2-1), yet a significantly reduces propranolol response (Figure 2-2, Figure 2-3). Importantly, we have shown that pravastatin significantly increases the potency of propranolol to prolong PR interval, a measure of β-adrenergic antagonism. The mechanism of interaction between propranolol and pravastatin, which is observed in both healthy and inflamed rats, appears to be at the pharmacodynamic level since pravastatin did not influence the pharmacokinetics of propranolol. Neither did the extent of propranolol binding to plasma proteins change.

2.6.1. Involvement of inflammation in cardiovascular disease

Inflammation is an integral part of cardiovascular diseases.[50, 315] In addition, rheumatoid arthritis and cardiovascular disease patients have overlapping inflammatory mediators profiles.[91] This may, at least in part, explain why rheumatoid arthritis patients have higher cardiovascular mortality than does the general population.[68] Literature supports a T_H1 (pro-inflammatory) to T_H2 (anti-inflammatory) ratio imbalance in both cardiovascular diseases and rheumatoid arthritis,[21, 316] and thus the two diseases are similar mechanistically. It is likely, therefore, of therapeutic significance that some cardiovascular drugs demonstrate diminished potency in inflammatory conditions.[91]

2.6.2. Effects of statins on inflammatory status and drug response

The observed effect of the hydroxymethylglutaryl-CoA reductase inhibitor on the β-adrenergic receptor response may be associated with alteration of T_H1 and/or T_H2 activities. Indeed, statin administration has been shown to both reduce pro-inflammatory IFN-γ[287] and elevate anti-inflammatory IL-10.[287, 317] In the present study pravastatin administration was, indeed, associated with a lowering of already elevated serum concentration of IFN-γ in inflamed rats (Figure 2-1). This may suggest an association between the cytokine and the β-adrenergic receptor responsiveness. However, the increased β-adrenergic receptor responsiveness was also noticed in the healthy rats (Figure 2-3). These rats, however, demonstrated serum IFN-γ concentrations that were so low that the concentration could not be measured using the available assay (Figure 2-1). In addition, pravastatin treatment increased the response to propranolol in inflamed rats (Arthritis/Pravastatin vs. Healthy/ Placebo groups, Figure 2-3). This occurred despite the observed and still significantly greater IFN-γ concentration in the former inflamed group as compared to the healthy groups (Figure 2-1). These observations render the possible association between serum IFN-γ concentrations and β-adrenergic receptor more complex, if not questionable or incidental.

Neither inflammation nor statin treatment significantly influenced serum concentrations of the anti-inflammatory cytokine, IL-10 (Figure 2-1). This may be attributed to the observed great inter-animal variations (Figure 2-1) and/or the nature of the animal model used and the short duration of pravastatin treatment relative to the 5-13 days reported in other experiments.[317-319]

In our study we chose to measure IFN-γ concentration as a marker of T_H1 cell activity because it is a hallmark T_H1 cytokine and it counters the effect and development of T_H2 cells.[17, 320] We measured IL-10 as a marker of T_H2 cell activity because of its strong ability to suppress T_H1 cells[17] and because its concentration is up-regulated by statins.[317] Additionally, IFN-γ is elevated in rheumatoid arthritis patients whereas there is a relative deficit of IL-10.[81, 321] Pre-AA resulted in significant elevations of IFN-γ; however, IL-10 was not influenced by adjuvant injection. We also determined NO_X^- and CRP concentrations as two commonly used measures of inflammation, which are both elevated in rheumatoid arthritis.[4, 322] Pre-AA resulted in significant elevations in plasma NO_X^- but did not influence serum CRP. Healthy rat plasma differs from human in CRP concentration and function. Rat plasma contains substantially greater concentrations of CRP[79, 323] (approximately 400 μg/mL) as compared with humans[324] (approximately 1.5 μg/mL), indicating that a rise in CRP may not be as detrimental to the rat as has been suggested for humans. CRP is thought cause damage in human myocardial tissue via the activation of complement,[325] the innate part of the immune system that helps clean up pathogens or necrotic/apoptotic cells.[326] CRP does not, after all, activate complement in the rat, whereas in human it does.[323]

Pravastatin may reverse the down-regulating effect of inflammation on propranolol response through several mechanisms. They include, for example, the reversal of the following observations: (1) reduced drug-receptor binding that has been observed in both adjuvant arthritis and IFN-induced acute inflammation,[327] (2) diminished receptor density that has been observed in adjuvant arthritis, though not in IFN-induced acute inflammation[176, 327] (3) altered second messenger systems,[6] (4) a reduced β-AR responsiveness occurring independent of cAMP-related mechanisms,[328] (5) reduced receptor sensitivity secondary to stimulation of β_1-AR by norepinephrine,[166] where statins appear to normalize sympathetic outflow.[299] It is unlikely, in this study, that the effect of pravastatin on propranolol response is related to lipid levels since a similar dose of pravastatin for 4 weeks has been shown to have no effects on cholesterol levels in normolipidemic rats.[329]

2.6.3. Effects of inflammation and pravastatin administration on propranolol pharmacokinetics

Elevated propranolol concentrations in the inflamed rat (Figure 2-4) have been attributed to decreased hepatic clearance and/or increased concentration of the acute phase protein α-1-acid glycoprotein, hence, increased extent of drug-protein binding.[110, 112, 330] Pravastatin did not influence the elevated S- or R-propranolol concentrations observed in inflamed rats. Nor did it alter the protein unbound fraction of the drug (Table 2-1). Our expectations included at least a trend toward normalization of propranolol clearance in response to the statin therapy. Elevated pro-inflammatory mediators such as IFN-γ[331] and nitric oxide,[79, 332] which are associated with reduced intrinsic clearance of the cytochrome P-450 enzyme system, were expected to be reduced by pravastatin.[287, 333] The observed elevated concentrations of IFN-γ, but not NO_X^-, were reduced in inflamed rats that were treated with pravastatin. This may suggest that our duration of statin therapy has not been sufficiently long, and/or the pro-inflammatory mediator(s) responsible for diminished clearance of propranolol has not been affected by pravastatin.

In addition, the response to propranolol in healthy rats has been observed to weaken after surgical intervention. It was thought that a reduced availability of the protein-unbound drug concentration secondary to increased concentration of the acute phase protein, α-1-acid glycoprotein would reduce response.[116] However, our data show that protein binding was not a determinant of propranolol response, whereas inflammatory status may be partly a determinant.

2.7. *Conclusions*

Inflammation has been recognized as a risk factor in cardiovascular diseases such as myocardial infarction.[334] In addition, inflammation appears to down-regulate some cardiovascular receptors.[4] It is plausible that drug non-responsiveness to certain cardiovascular drugs may contribute to the morbidity and mortality of patients afflicted with inflammatory diseases. The cholesterol-independent effects of statins may arise from the anti-inflammatory properties of these drugs.[50] Nevertheless, according to the data generated from the animal model used in this study, pravastatin appears to reverse the down-regulatory effect of inflammation on β-adrenergic receptors. This is important since, once confirmed in humans, the observation is likely to have important therapeutic consequences for inflammatory conditions that contribute to drug non-responsiveness.

2.8. *Acknowledgements*

Dr. Ali Aghazadeh for technical assistance with HPLC. John Clements, is a Canadian Institutes for Health Research (CIHR) Strategic Training Fellow in TORCH (Tomorrow's Research cardiovascular health Professionals). We would like to acknowledge TORCH, CIHR, the Alberta Heritage Foundation for Medical Research, and the Heart and Stroke Foundation of Canada.

3. Adjuvant Arthritis-Associated Inflammation Down-Regulates Norepinephrine Transporter and β1-Adrenergic Receptors in Rat Heart

3.1. *Introduction*

Inflammatory mediators affect the density and/or function of L-type calcium channels, β_1-ARs, or potassium channels.[4, 82, 114, 124] However, there is not a strong correlation between β1-ARs pharmacodynamic response and inflammatory mediator concentration in Pre-AA. Additionally, pravastatin normalizes the response to propranolol in Pre-AA, despite the concentration of IFN-γ is higher in inflamed rats than in non-inflamed rats. Identifying markers of inflammation, such as the MMPs, that strongly predict drug response and drug concentrations would be clinically beneficial. Characterizing cardiac sympathetic nervous activity in Pre-AA rats would also be of benefit, since excessive sympathetic activity in the heart is a component of rheumatoid arthritis, possibly contributing to excessive cardiovascular mortality.[69, 76]

3.2. *Hypotheses*

A. Excessive sympathetic activity in Pre-AA rats, as measured by altered norepinephrine concentration and norepinephrine transporter density, contributes to diminished PR interval response.
B. Matrix metalloproteinase activity in the heart is highly correlated with both inflammatory mediators and reduced PR interval response to propranolol, and pravastatin has a normalizing effect.
C. Alterations of β_1-AR signaling in Pre-AA rats are normalized by pravastatin administration.

3.3. *Objectives*

A. Inflammatory profile of Pre-AA rats and the effects of pravastatin

    a. To characterize MMP-2 and MMP-9 activity in the Pre-AA animal model.
    b. To determine how pravastatin alters MMP-2 and MMP-9 activity in the Pre-AA animal model.

B. β_1-adrenergic receptor signaling changes
    a. To characterize cardiac norepinephrine concentration and norepinephrine transporter density.
    b. To assess critical proteins involved in β_1-AR signal transduction in Pre-AA inflammation in rats following pravastatin administration. These proteins include β_1-AR, G-protein-coupled receptor kinase-2, and the L-type calcium channel.

3.4. *Methods*

3.4.1. Animals

This study was approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. The animals were housed in a 12 h light/dark cycle and always had free access to water. The animal tissues used in this study originated from rats of the pharmacodynamic section of the study published by Clements and Jamali, 2007.[82] These animals, before having any invasive procedures, were deeply anesthetized using a mixture of halothane and oxygen.

3.4.2. Materials

Protease inhibitor cocktail for mammalian tissues (#P 8340) was obtained from Sigma-Aldrich (St. Louis, MO). Tris (0.025M)/glycine (0.192 M) buffer, and Tris (0.025 M)/glycine (0.192 M)/SDS (0.1%) buffer were purchased from ICN Biomedicals (Aurora, Ohio). Immun-Star HRP Chemiluminescent Kit, Precision Plus Protein Standards Dual Color, Bio-Rad D_C Protein Assay Sodium, dodecyl sulfate, TEMED, 10% Tween 20, and 40% acrylamide/Bis 29:1 solution were purchased from Bio-Rad (Hercules, CA). Pravastatin sodium was acquired from Apotex (Toronto, Canada). Labor LDN Noradrenaline Research EIA (enzyme immunoassay) kit was obtained from Rocky Mountain Diagnostics (Colorado Springs, CO, USA). Heat-killed desiccated *Mycobacterium butyricum* was purchased from Difco Laboratories (Detroit, MI).

3.4.3. Antibodies

Rabbit monoclonal (Y137) to βARK1 (GRK-2) (#ab32558) and mouse monoclonal to beta actin-loading control (#ab8226) was purchased from Abcam Incorporated (Cambridge, MA). Rabbit anti-norepinephrine transporter (I.D. norepinephrine transporter-101AP) was purchased from FabGennix Inc. International (Frisco, Texas). Rabbit polyclonal to β_1-AR (#PA1-049) was purchased from Affinity BioReagents (Golden, CO) Rabbit anti-L-type calcium channel (α_{1C} subunit) (#C 1603) was purchased from Sigma. Immun-Star goat anti-mouse (GAM)-HRP conjugate and Immun-Star goat anti-rabbit (GAR)-HRP conjugate was purchased from Bio-Rad (Hercules, CA).

3.4.4. Study Design

We recovered plasma and heart samples from an experiment in rats that investigated the effects of Pre-AA-associated inflammation and pravastatin administration on propranolol pharmacodynamics, propranolol pharmacokinetics, and on serum or plasma concentrations of NO_X^-, CRP, IFN-γ, and IL-10.[82] The current study attempts to show that propranolol pharmacodynamic response, in inflamed animals, is decreased despite the fact that propranolol concentrations are elevated as a result of slower clearance. The study also attempts to determine, by studying molecular mechanisms, how pravastatin administration was able to restore propranolol response. For a detailed description of the study design of Clements and Jamali 2007[82] or to Chapter 2 of this thesis.

Briefly, adult male Sprague-Dawley rats were divided into 4 groups including Healthy/Placebo, Healthy/Pravastatin, Arthritis/Placebo, and Arthritis/Statin. Inflammation was induced by injection of placebo (normal saline) or *Mycobacterium butyricum* in squalene into the base of the tail. On Days 4-8, animals received either oral pravastatin 6 mg/kg twice daily or placebo (vehicle).

The current study uses the hearts and plasma of these rats, which have been stored at -70°C.

There were 63 heart and plasma samples stored from the previous study. For this study we analyzed 48 (n=12) and 24 (n=5-6) randomly selected hearts for MMP-9 and MMP-2 gelatin zymography, respectively, 32 (n=8) randomly selected hearts for western blot analysis, and 30 (n=7-8/group) randomly selected hearts and plasma samples for norepinephrine analysis.

3.4.5. Western Blot

Western blot analysis was use to determine the density of norepinephrine transporter, β_1-AR, GRK-2, and the α_{1c} subunit of the L-type calcium channel ($Ca_v1.2$) (detecting both high and low molecular weight $Ca_v1.2$). Although there are 10 L-type calcium channel-α_1 subunits, only $Ca_v1.2$ is found in high levels in the heart.[335] β-actin was used as a loading control for all but the $Ca_v1.2$. Instead, for $Ca_v1.2$, a non-specific binding band was used to assure equal loading. Whole heart was triturated under liquid nitrogen. Approximately 75 mg of ground heart was diluted 1 in 5 with homogenizing buffer (Tris Buffer pH 7.4, 0.05 M, 2% protease cocktail inhibitor), followed by 1 min of trituration on ice. After centrifugation for 1 min, the supernatant was kept and assessed for protein concentration using the Lowry method. Heart protein samples (45 µg for GRK-2, and 60 µg for norepinephrine transporter, β_1-AR, and $Ca_v1.2$) were prepared with Lane Marker Reducing Sample Buffer®, followed by submersion of the sample container in boiling water (10 sec for $Ca_v1.2$, 4 min for GRK-2, and 1 min for norepinephrine transporter, and the β_1-AR). Proteins were separated using SDS-polyacrylamide electrophoresis (constant voltage). A 7.5% SDS-polyacrylamide gel was used to separate norepinephrine transporter, β_1-AR, and GRK-2, whereas a 6.5% gel was used to separate $Ca_v1.2$. Wet-transfer blotting was performed using a nitrocellulose membrane (0.45 µm, Trans-Blot Transfer Medium). Non-specific antibody binding was minimized by blocking for 2 hours at room temperature or overnight at 4°C in blocking solution (5% skim milk/ 2% bovine serum albumin/ 0.05% Tween in 25 mM tris-buffered saline, pH 7.4). The nitrocellulose membranes were then incubated with primary antibody diluted in 0.05% Tween in 25 mM tris-buffered saline, pH 7.4 at the following concentrations; norepinephrine transporter 1:750, β_1-AR 1:1000, GRK-2 1:720, and $Ca_v1.2$ 1:720. Horseradish peroxidase-conjugated secondary antibodies (goat anti-rat or goat anti-mouse), were diluted 1:15000 in blocking solution then incubated at room temperature for 1 hour. Secondary antibodies were visualized using a chemiluminescense kit (Immun-Star HRP Chemiluminescent Kit) and the image was captured using Kodak BioMax Light Film. Bands were assessed by densitometry using Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA). The ratio of the density of the band of interest to loading control (β-actin) accounted for variations in sample loading.

3.4.6. MMP Gelatin Zymography

MMP gelatinase activity was assessed using a previously described zymography method.[336] Briefly, triturated heart tissue was homogenized in a 5 times dilution of homogenizing buffer (50 mM Tris-HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mM dithiothreitol, 10 µg/mL leupeptin, 10 µg/mL soybean trypsin inhibitor, 2 µg/mL aprotinin, and 0.1% Triton X-100), followed by centrifugation and collection of supernatant. The supernatant was then assessed for protein concentration using the Bio-Rad protein assay. 30 µg of protein for the MMP-2 assay, or 20 µg of protein for the MMP-9 assay, was loaded onto a 7.5% polyacrylamide gel that was copolymerized with 2 mg/ml of gelatin. After electrophoresis, the gels were rinsed in 2.5% Triton X-100 for 20 minutes three times, then two more times briefly in incubation buffer consisting of 50 mM Tris-HCl, 5 mM $CaCL_2$, 150 mM NaCl and 0.05% NaN_3. Gels were then incubated at 37 °C for 48 hours (MMP-2) or 72 hours (MMP-9) in incubating buffer. After the incubation period, the gels were stained for 4 hours in 2% Coomassie Brilliant blue, 25% methanol, and 10% acetic acid. The gels were then de-stained for 1 hour with 2% methanol/ 4% acetic acid. The gel zymograms were scanned using Sigmagel software, and MMP activities were expressed in arbitrary units standardized against HT-1080 cell conditioned medium internal standard (MMP-

2) or reference sample internal standard (MMP-9) (ratio of the density of the band of interest to a sample which was chosen as an internal standard and was loaded into each gel).

3.4.7. Norepinephrine Competition ELISA

Norepinephrine concentration was determined using a competitive ELISA system. Plasma samples were added directly to the 96 well plate. For whole heart samples, 150 µl of ice cold 0.01 N hydrochloric acid was added to 100 µg of triturated tissue. The triturated tissue was homogenized on ice using the Pellet Pestle for 1 min. After 5 min of centrifugation at 19 000 x g at 4°C, 100 µl of supernatant was added to the norepinephrine enzyme immunoassay 96 well plate. The following simplified steps were applied to the immunoassay plate as per the kit instructions: a) norepinephrine was extracted using a cis-diol specific affinity gel, b) acetylation to N-acylnorepinephrine, c) enzymatic conversion to N-acylnormetanephrine, d) acetylated norepinephrine from the sample and solid-phase well-bound norepinephrine competed for a fixed number of antiserum binding sites, e) upon system equilibration, free acetylated norepinephrine, which was derived from the sample, and free acetylated norepinephrine-antiserum complex complexes were removed from the wells, f) the antibody that was bound to the kit's norepinephrine (solid phase) is detected using an anti-rabbit IgG-peroxidase conjugate using the substrate tetramethylbenzidine (TMB). Using a plate reader at 450 nm gives a reading inversely proportional to the norepinephrine content of the sample since the peroxidase is bound to the solid phase norepinephrine.

3.4.8. Statistics

Statistical analyses were performed in Statistical Analysis Systems (SAS) version 9.1 (SAS institute, USA). For norepinephrine transporter, β_1-AR, $Ca_v1.2$, GRK-2, MMP-2, and MMP-9, ANOVA analyses, with Tukey p-value adjustments for multiple comparisons, were conducted using Proc GLM (generalized linear model procedure) with gels set to block. Correlation analyses were performed in the Analyst program, and correlation plots were created in Proc CORR (correlation procedure).

Norepinephrine statistics were set up for two pre-planned t-test comparisons: healthy versus inflamed animals, and inflamed animals versus inflamed/pravastatin animals. The data was tested for homogeneity of variance using an F-test then compared using an unpaired two-tailed T-test for samples of unequal variance for plasma norepinephrine, and an unpaired two-tailed T-test for heart norepinephrine.

Data is presented as the mean±SEM. p-Values that were less than 0.05 were considered significant. Correlation analyses r-values were considered significant when p-values were less than 0.05.

3.5. *Results*

3.5.1. Western Blot

Compared to healthy rat hearts, Pre-AA rat hearts had lower norepinephrine transporter density (Figure 3-1) and β_1-AR density (Figure 3-2). Figure 3-3 demonstrates the statistically significant parallel reduction ($r=0.978$, $p<0.0001$) between norepinephrine transporter denstiy and β_1-AR density in placebo and inflamed animals. This strong correlation exists even when all animals are included ($r=0.971$, $p<0.0001$). Pravastatin administration elicited no effects.

GRK-2 and the 190 and 210 kDa $Ca_v1.2$ 190 subunit densities all were not influenced by inflammation or pravastatin treatment (Table 3-1).

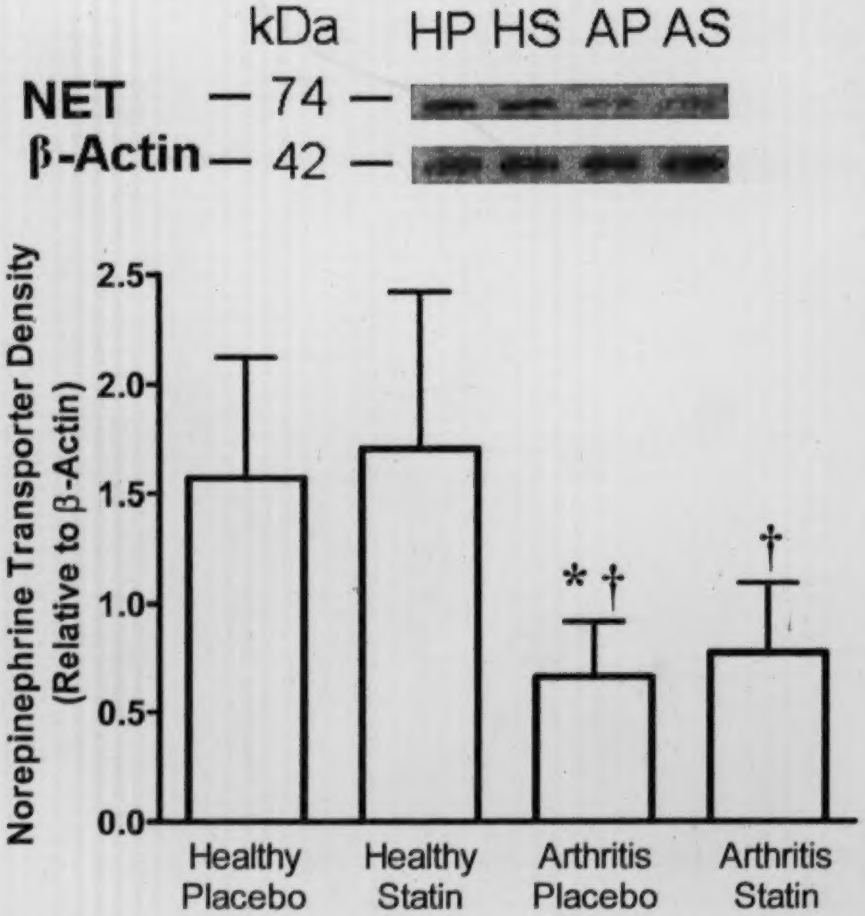

Figure 3-1. The effect of Pre-AA inflammation, 8 days after the injection of *Mycobacterium butyricum*, on norepinephrine transporter density in whole heart tissue homogenate in the presence or absence of pravastatin treatment on days 4 to 8(n=8/group, mean±sem). Inset is a sample of a western blot that was used in the analysis.

*Significantly different compared to Healthy/Placebo, $p<0.05$.

†Significantly different compared to Healthy/Statin, $p<0.05$.

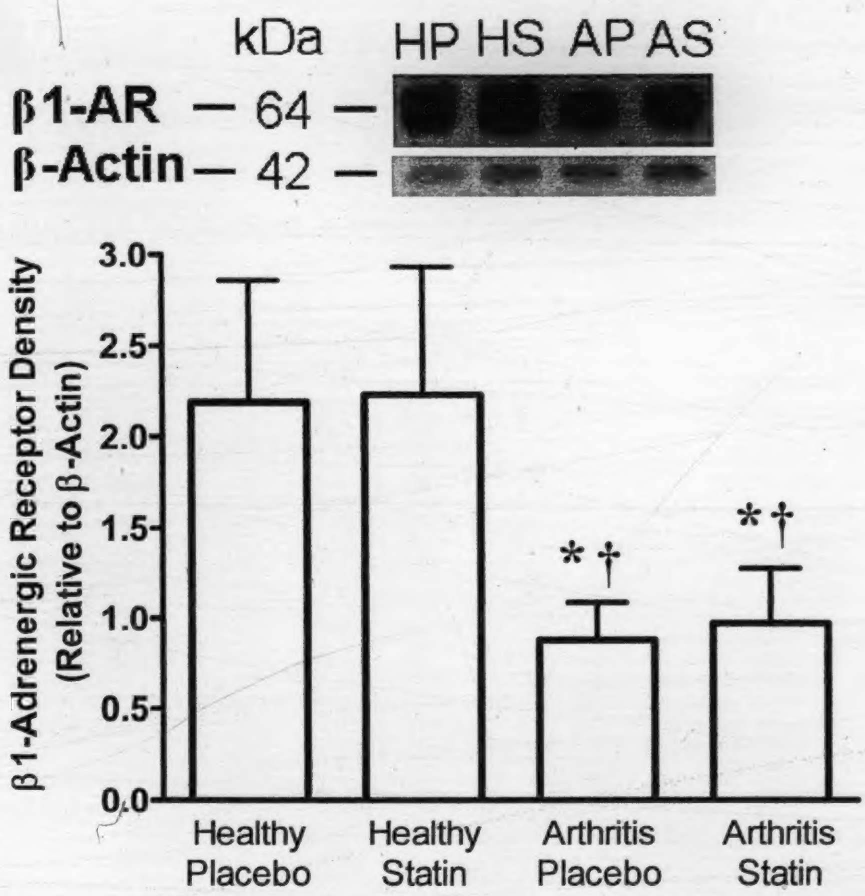

Figure 3-2. The effect of Pre-AA inflammation, 8 days after the injection of *Mycobacterium butyricum*, on β1-adrenergic receptor density in whole heart tissue homogenate in the presence or absence of pravastatin treatment on days 4 to 8(n=8/group, mean±sem). Inset is a sample of a western blot that was used in the analysis.

*Significantly different compared to Healthy/Placebo, $p<0.05$.

†Significantly different compared to Healthy/Statin, $p<0.05$.

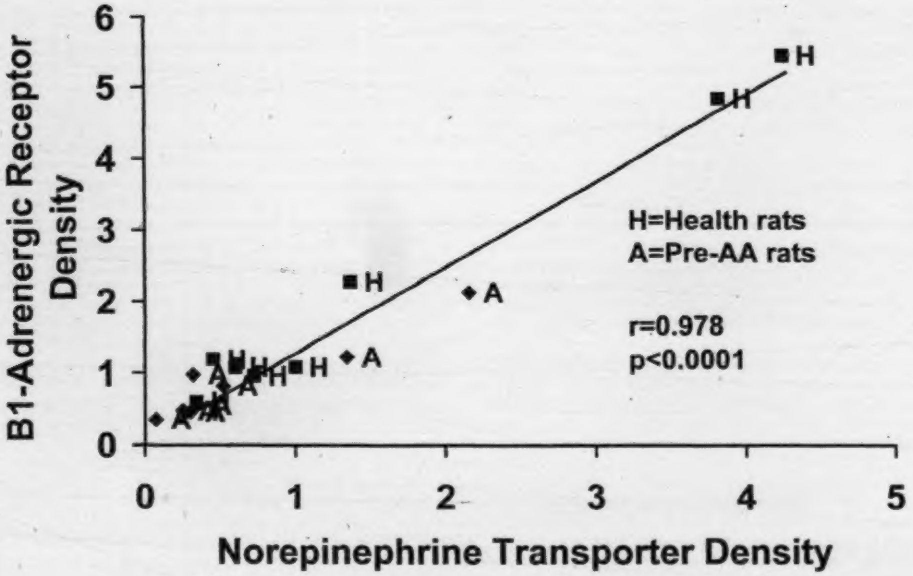

Figure 3-3. The correlation between β1-adrenergic receptor and norepinephrine transporter density in whole heart homogenates in both healthy and Pre-AA rats (H=Healthy rats and A=Day 8 Pre-AA rats) Pravastatin treated rats have not been included, though their inclusion increases the correlation coefficient. (n=16).

Table 3-1. Effect of Pre-AA 8 days after *Mycobacterium butyricum* injection and pravastatin treatment for 4 days on select proteins found in homogenized rat heart. Western blot data (Ca$_v$1.2 and GRK-2) are presented in terms of optical density. 72-kD MMP-2 activity is presented as relative zymographic activity relative to internal standard. No significant differences were found, and the means are presented ±SEM.

	Healthy/Placebo	Healthy/Statin	Arthritis/Placebo	Arthritis/Statin
Ca$_v$1.2 190 kDa	2.51 ± 0.48	2.7 ± 0.91	1.46 ± 0.20	2.72 ± 0.58
Ca$_v$1.2 210 kDa	2.13 ± 0.35	2.14 ± 0.58	1.64 ± 0.32	2.28 ± 0.25
GRK-2	0.94 ± 0.07	0.99 ± 0.07	1.00 ± 0.09	1.07 ± 0.07
72 kDa MMP-2	1.17 ± 0.09	1.14 ± 0.12	0.94 ± 0.09	1.05 ± 0.08

3.5.2. MMP Gelatin Zymography

Inflammation significantly elevated 92 kDa MMP-9 activity, whereas pravastatin treatment had no influence (Figure 3-4). 72 kDa MMP-2 represents the proMMP form that is activated by oxidative stress in the absence of enzymatic cleavage.[337] 72 kDa MMP-2 activity was not influenced by either Pre-AA or pravastatin treatment (Table 3-1).

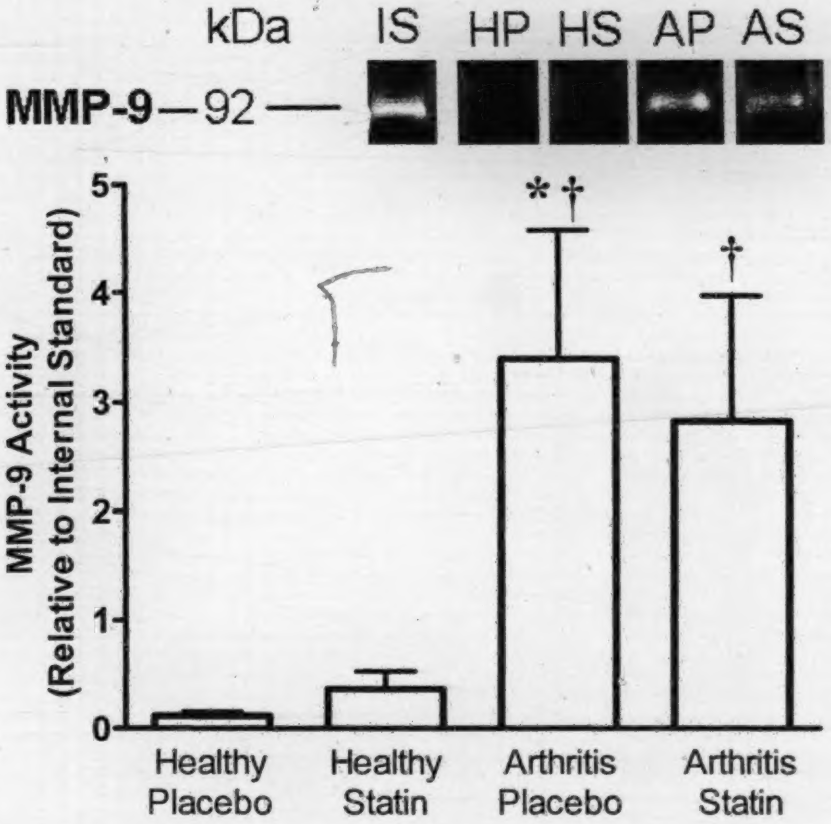

Figure 3-4. The effect of Pre-AA 8 days after *Mycobacterium butyricum* injection on whole heart MMP-9 activity in the presence or absence of pravastatin treatment for 4 days. Inset is an MMP-9 gelatin zymography example that demonstrates internal standard and MMP-9 activity (n=12/group).

[*] Significantly different compared to Healthy/Placebo, $p<0.05$.

[†] Significantly different compared to Healthy/Statin, $p<0.05$.

3.5.3. Norepinephrine Analysis

Pre-AA animals compared to healthy controls had no difference in plasma or cardiac norepinephrine content (Healthy/Placebo vs. Arthritis/Placebo; plasma norepinephrine 2.3±0.6 vs. 1.1±0.3 ng/ml; or cardiac norephinephrine 9.8±1.6 vs. 9.2±1.6 ng/ml). Additionally, pravastatin treatment in Pre-AA animals did not influence plasma or cardiac norepinephrine concentration (Arthritis/Placebo vs. Arthritis/Statin plasma 1.4±0.4 vs. 2.3±0.9 ng/ml; and cardiac 7.6±1.2 vs. 7.2±1.7 ng/ml).

3.5.4. Correlation Analyses

MMP-2 activity and 190 kDa Ca$_v$1.2 density were significantly correlated (r=0.649, p=0.0008). In inflamed animals (pooling pravastatin-treated animals with control animals), propranolol response (maximum PR interval) and Ca$_v$1.2 190 density were significantly correlated (r=0.558, p=0.025) (Figure 3-5). Also, pooled placebo and inflamed animals, exhibit a significant correlation between 210 kDa Ca$_v$1.2 density and norepinephrine transporter density (r=0.630, p=0.009). Pooled placebo and inflamed animals also exhibit a significant correlation between 210 kDa Ca$_v$1.2 density and β_1-AR density (r=0.596, p=0.017).

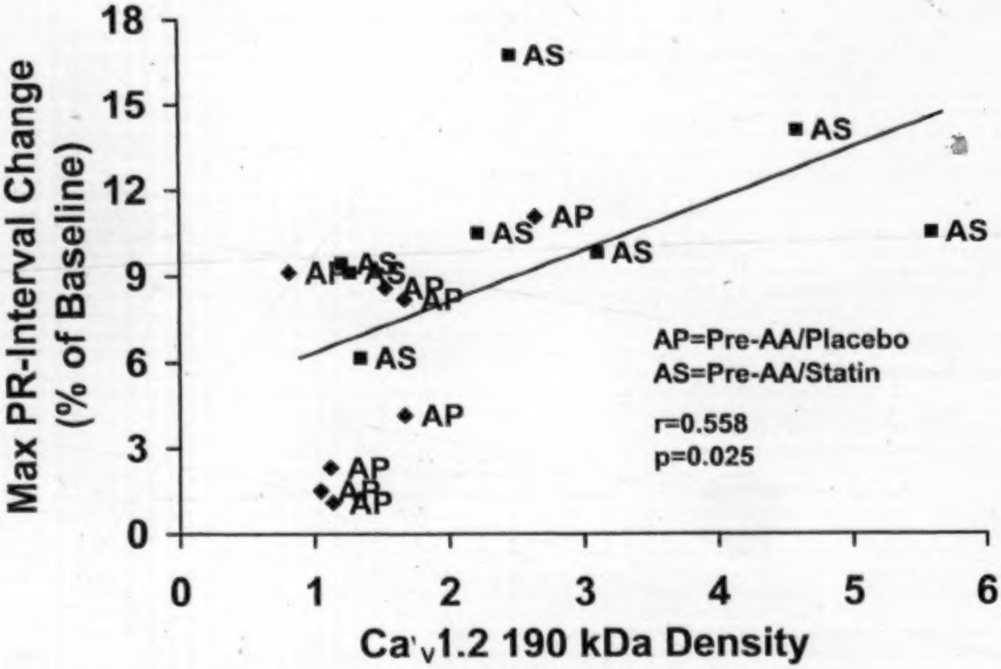

Figure 3-5. The correlation between maximum PR interval response to propranolol and 190 kDa Ca$_v$1.2 density in whole heart homogenates of Pre-AA rats 8 days after *Mycobacterium butyricum* injection. AP=Pre-AA/Placebo, and AS=Pre-AA/Statin (n=16).

3.6. *Discussion*

Presented here are novel findings, in the Pre-AA rheumatoid arthritis model in rats, that β_1-AR density is reduced in parallel with norepinephrine transporter density. Alterations in cardiac sympathetic activity may therefore be implicated in the pharmacotherapeutic desensitization process that occurs in inflammatory conditions. This is the first report that claims experimental arthritis is associated with reductions in norepinephrine transporter density.

3.6.1. Norepinephrine transporter and β_1-adrenergic receptor

The strong correlation between norepinephrine transporter density and β_1-AR density, and the absence of a correlation between inflammatory mediators and β_1-AR density, helps establish a contributory role for reduced norepinephrine transporter density subsequently β_1-AR density. Though this information is circumstantial, it is important since it was previously thought that inflammation reduces cardiac response primarily as a function of inflammatory mediator interaction with the β_1-AR signaling pathway.[6, 114, 338] Perhaps persistent agonism of the β_1-AR by norepinephrine reduces β_1-AR density, and ultimately attenuates PR interval response to propranolol.

Excessive sympathetic nervous system activity is characteristic of rheumatoid arthritis, and it may contribute to excessive cardiovascular mortality.[76, 185, 218-220] Norepinephrine transporter functionality helps dictate the distribution of sympathetic innervation and activity.[339] Inflammation influences sympathetic outflow by interacting with the brain, specifically in the rostral ventrolateral medulla.[340] Statins reduce sympathetic nervous system outflow in rabbit by inhibiting inflammatory pathways that produce superoxide in the brain.[341] 24-h urinary norepinephrine excretion is also decreased by statin use in stroke-prone, spontaneously hypertensive rats.[342] We expected the pravastatin-treated animals to have normalized norepinephrine transporter density, however, no differences were observed, possibly due to the short duration of pravastatin treatment.

Norepinephrine has anti-inflammatory properties, and its release may help limit the severity of inflammation. For example, norepinephrine in lymphoid tissues decreases TNF-α production by immune cells.[343] Little is known about the regulation of norepinephrine transporter in inflammatory conditions, however: a) IFN-α administration to adrenal medullary cells causes reductions in norepinephrine transporter density and reduced [H^3]norepinephrine uptake,[243] b) IL-1β is able to stimulate the release of norepinephrine from sympathetic neurons,[244] and c) NO inhibits the uptake of norepinephrine.[206] Other cytokines including IL-6 and TNF-α are seemingly not involved in the regulation of catecholamine synthesis or re-uptake.[244]

The main limitation of this study is not having measured local post-synaptic norepinephrine concentration or norepinephrine turnover rates. This is because measuring the turn-over of norepinephrine would give a better indicator of sympathetic activity.

3.6.2. MMP-2, MMP-9 and the L-type calcium channel

We note in inflamed rats a significant positive correlation between MMP-2 activity and 190 kDa $Ca_v1.2$ density. MMP-2 is known to have several intra-myocardiocyte targets including troponin I, myosin light chain-1, and α-actinin.[337] Consequently, heightened MMP-2 activity decreases myocardial contractility,[344] and impairs recovery from ischemia-reperfusion injury.[345] The $Ca_v1.2$ is resistant to a number of proteases including trypsin, subtilisin, proteinase K, carboxypeptidase, papain, and thermolysis,[346] though it is cleaved by calpain.[347] The correlation between 190 kDa $Ca_v1.2$ density and MMP-2 activity suggests that the 210 kDa $Ca_v1.2$ subunit of the L-type calcium channel may be a target for MMP-2, causing the formation of the lower weight 190 kDa $Ca_v1.2$ subunit. This is only suggestive data needing further investigation.

As 190 kDa $Ca_v1.2$ density increased in inflamed animals, so to did the maximum PR interval prolongation (Figure 3-5). In fact, the 190 kDa $Ca_v1.2$ protein is the only protein that was significantly correlated with propranolol response. Perhaps this is because $Ca_v1.2$ is part of the L-type calcium channel.

The L-type calcium channel is the final protein in the β_1-AR pathway that causes changes in PR interval. The β_1-AR/$Ca_v1.2$ pathway is mediated by the β-adrenergic/cAMP signaling pathway.[348] The carboxyl tail of the $Ca_v1.2$ inhibits Ca^{2+} current through the pore of the $Ca_v1.2$ subunit. The cleaved portion can block Ca^{2+} current despite its cleaved state.[349] Recently it has been shown in cardiomyocytes that ryanodine receptors interact with the mobile cytoplasmic tail of the $Ca_v1.2$, and thereby help synergize calcium-induced calcium release,[350] but how this system works with the cleaved cytoplasmic tails of $Ca_v1.2$ is uncertain. Regarding the phosphorylation status of the $Ca_v1.2$, only the higher molecular weight protein is effectively phosphorylated by protein kinase A, and thereby allowing Ca^{2+} entry.[347] Ultimately, this issue is complicated by the fact that the cleaved cytoplasmic tail of the $Ca_v1.2$ can still interact with the L-type calcium channel.

IFN-γ plays an important role as the hallmark T_H1 cytokine in inflammatory models such as Pre-AA.[13, 82, 320] IFN-γ influences many biochemical pathways, including the activation of MMP-9.[351] How elevated MMP-9 interacts with propranolol responsiveness is less clear as we found no significant associations between MMP-9 and norepinephrine transporter, β_1-AR, $Ca_v1.2$, or PR interval response. MMP-9 is found in high levels in leukocytes,[337] so perhaps cardiac leukocyte infiltration elevated MMP-9 concentration in the whole heart tissue preparation that we used.

3.6.3. G-protein-coupled receptor kinase-2

We expected GRK-2 density to be up-regulated in inflamed animals because arthritis is associated with increased sympathetic activity. Increased sympathetic activity causes chronic stimulation of the β_1-AR, thereby increasing GRK-2 density via homologous desensitization mechanisms. As shown in Table 3-1, GRK-2 density was not upregulated, possibly because inflammatory mediators annulled this effect. Inflammation has been shown to decrease activity in the GRK-2 promoter site.[352] Also, inherent limitations in our technique preclude us from ruling out changes in the active phosphorylated form of GRK-2.

3.7. *Conclusions*

We provide novel information that suggests inflammation alters cardiac sympathetic tone by reducing norepinephrine transporter density. Consequently, elevated concentration of synaptic norepinephrine may downregulate β_1-AR pharmacodynamic responsiveness. Previously it was thought that inflammation directly down-regulated β_1-ARs and down-stream signal transduction mediators. Rather, inflammation may also exert, in tandem, changes in sympathetic activity and direct inflammatory mediator effects on cellular machinery. This work highlights the need for in vivo cardiovascular pharmacotherapy research to be cognizant of the effects of, not only direct effects of inflammation, but also the interplay between inflammatory mediators and peripheral neurons or the brain, which subsequently alter sympathetic outflow. A preliminary experiment investigating the effects of sympatholysis and atorvastatin administration on cardiovascular responsiveness to isoproterenol is detailed in Chapter 5 (Appendix of miscellaneous experiments).

3.8. *Acknowledgements*

We would like to sincerely thank Jolanta Sawicki for her expert technical assistance in processing MMP-9 samples. At the time these experiments were conducted JD Clements was a Canadian Institutes for Health Research (CIHR) Strategic Training Fellow in Tomorrow's Research cardiovascular Health Professionals (TORCH). We would like to acknowledge TORCH, CIHR, the Alberta Heritage Foundation for Medical Research, and the Heart and Stroke Foundation of Canada.

4. General conclusions

Rheumatoid arthritis is a disease of complex pathophysiology. The inflammatory changes that disfigure joints, and cause so much pain, are also involved in extra-articular effects of rheumatoid arthritis. Cardiovascular changes that accompany rheumatoid arthritis contribute to excessive morbidity and mortality in this group of patients. Effective risk-reduction strategies for cardiovascular diseases are potentially undermined by the fact that inflammation reduces pharmacodynamic response to certain cardiovascular drugs.

Assessing drug exposure by measuring either the maximum concentration or area under the concentration curve is generally useful for predicting pharmacodynamic response. However, inflammation can cause disjuncture between pharmacokinetic and pharmacodynamic profiles of drugs that have been administered to patients who have underlying inflammation. Rheumatoid arthritis, obese, Crohn's disease, or just being elderly, can reduce cardiovascular pharmacodynamic response; in part because these conditions are of an inflammatory nature.

Normalizing pharmacodynamic response to cardiovascular drugs in rheumatoid arthritis patients is a challenge, which if successful, may have wide ranging application in cardiovascular risk reduction programs. Discovering a method or agent that restores pharmacodynamic responsiveness would be of clear benefit. The statins have been proven safe and effective at reducing the risk of morbidity and mortality in hypercholesterolemia and many other cardiovascular conditions. Given the wide range of their anti-inflammatory pleiotropic effects, the use of statins in rheumatoid arthritis patients may prove useful for normalizing the drug response.

This thesis describes the background and rationale necessary for justifying our experimental approach. I then tried to determine whether or not pravastatin normalizes the response to propranolol in inflamed rats. If pravastatin was to normalize propranolol response, then what mechanisms could be involved? More importantly, I have designed this experiment such that the information was readily transferable to the clinical setting.

The metabolism of drugs is generally slowed down in the presence of inflammation. This effect is not unique to any specific inflammatory disorder, but rather is present across the spectrum of inflammatory diseases. A number of mechanisms contribute to reductions in drug metabolism, including reduced enzyme function by nitric oxide inhibition, or reduced transcription and translation of metabolizing enzymes. Altered hepatic blood flow and protein binding may also contribute to drug metabolism changes in inflammatory conditions.[4] In combination, these effects can reduce the clearance of drugs to a degree that drug levels are significantly higher than in those subjects that do not have inflammation.

Rheumatoid arthritis, a common inflammatory disease, results in a significant elevation in inflammatory markers, including nitric oxide. Consequently, rheumatoid arthritis is capable of causing a significant increase in propranolol and verapamil concentration. The research in this thesis was facilitated by the use of an animal model of rheumatoid arthritis called Pre-AA. I confirmed that the Pre-AA animal model similarly increased circulating propranolol 10-fold, making it a good model for assessing pharmacokinetic changes in arthritis. Importantly, protein binding in Pre-AA was shown not to be different than control groups. These data are similar to other's findings whereby verapamil concentration is increased Pre-AA by many fold.[79, 83]

Diseases of inflammatory nature not only are capable of causing elevated drug concentration, but are also associated with reduced cardiovascular drug response. This occurs in rheumatoid arthritis patients, the obese, and in the elderly,[91, 119, 120] and potentially minimizes the risk reduction benefits of cardiovascular preventive therapies. Finding a treatment that could potentially reverse this effect was one of the objectives of this thesis.

Pravastatin, chosen because of its anti-inflammatory properties, was administered to Pre-AA rats in an attempt to see if it could reverse the down-regulating effects of inflammation on β_1-ARs. We also expected that pravastatin-treated Pre-AA animals would have normalized propranolol exposure. I demonstrated that pravastatin did not influence propranolol exposure in Pre-AA animals.

The experiments described in this thesis demonstrate that Pre-AA, like adjuvant arthritis, reduces propranolol's PR interval responsiveness. One of my most important findings was that pravastatin administration in Pre-AA reverses the reductions in propranolol response that are characteristically observed in Pre-AA. By allowing an animal to response in a normal range to propranolol, pravastatin administration could potentially be useful in reducing cardiovascular risk in rheumatoid arthritis patients. The findings of this study could easily be translated into clinical research because pravastatin is a relatively safe drug that is currently on the market drug.

For each individual rat in the propranolol-pravastatin study, we had complete sets of information for pharmacodynamics, mediators of inflammation, and proteins of interest. In trying to determine the best predictor of PR interval response we performed correlation analyses. Interestingly, amongst the measured parameters, L-type-calcium channel density was the best predictor of maximum PR interval responsiveness with propranolol administration ($r=0.558$, $p=0.025$). This may indicate that the L-type-calcium channel is a better indicator of propranolol response than is the β-AR. Subsequent investigations could prove fruitful if calcium influx and turnover was assessed, which could be used as a more direct measure of pharmacodynamic response.

Inflammation is associated with increased cardiovascular risk. CRP,[50] TNF-α,[353] and serum amyloid A[354] are all predictive of outcome in cardiovascular disease. Statins reduce inflammation, as measured by IFN-γ, CRP, TNF-α, IL-6, or serum amyloid A,[257, 354, 355] and statins also reduce the risk of cardiovascular disease-related death.[50] Therefore, pravastatin could potentially reduce inflammation associated with Pre-AA induction, and therefore also the down-regulating effects of inflammation on β_1-ARs.

I have shown that Pre-AA significantly increases cardiac MMP-9 and circulating nitrite and IFN-γ concentration. Confirming my hypothesis, pravastatin significantly reduced IFN-γ, the hallmark T_H1 cytokine that is pivotal in the development of inflammatory diseases of this nature.

In these studies, it was peculiar that pravastatin-treated Pre-AA animals responded to propranolol in the presence of higher than normal IFN-γ, even if IFN-γ were lower than animals not treated with pravastatin. Therefore, we inferred that another mechanism, other than just the direct effects of inflammation on cardiomyocytes, was involved in β_1-AR desensitization. Cardiac norepinephrine transporter density is a surrogate marker for sympathetic nervous system activity. Low levels of norepinephrine transporter could increase the concentration of synaptic norepinephrine, thereby causing β_1-AR downregulation.

Another critical finding was that norepinephrine transporter and β_1-ARs are down-regulated in parallel in Pre-AA. This is the first time that norepinephrine transporter density has been shown to be reduced in the hearts of animals with experimental arthritis. Since the correlation between these two proteins is strong, and since strong correlations do not exist between β_1-ARs and the inflammatory mediators that we measured, we conclude that the sympathetic nervous system in experimental arthritis might contribute to β_1-AR desensitization. Featured in the chapter of miscellaneous experiments (Chapter 5), is a preliminary experiment that looks at the effects of atorvastatin and 6-hydroxydopamine in isoproterenol response. This experiment may eventually be used for characterizing the sympathetic nervous system in cardiovascular non-responsiveness to isoproterenol. Please read that section for further details.

The use of animal models has helped the scientific community's understanding of human disease. However, due to ethical concerns, opportunities to reduce animal suffering must be actively pursued. Such an opportunity was presented when the point of interest for inflammation associated with adjuvant arthritis was within cardiovascular system. Ling and Jamali, 2005 developed the Pre-AA rat for precisely this reason.

The experimental results of this thesis further characterize Pre-AA. Specifically, five important features of Pre-AA have been discovered:

Pre-AA,

- reduces propranolol response.
- reduces cardiac β_1-AR density.
- reduces norepinephrine transporter density.
- is T_H1-skewed (IFN-γ), as is adjuvant arthritis and rheumatoid arthritis. That is to say, overall immunological balance of Pre-AA is similar to rheumatoid arthritis patients.
- elevates MMP-9 activity in the heart, and may therefore reduce cardiac function.

The Pre-AA rat model of inflammation is not well suited for studying arthritic pain simply because the animals are euthanized prior to the development of pain. The use of Pre-AA in inflammation research, therefore, is most appropriately used for studying the effects of inflammation on the cardiovascular system. This is true because the presence of symptomatic arthritis is not needed for studying the effects of TH1-skewed inflammation on pharmacodynamic responsiveness in the heart.

4.1. *Implications*

In this thesis we have assessed how pravastatin influences propranolol pharmacokinetics and pharmacodynamics in Pre-AA, and how it alters inflammatory mediators and proteins involved in β_1-AR signaling. We provide evidence that pravastatin normalizes propranolol pharmacodynamics but not pharmacokinetics. By showing that norepinephrine transporter and β_1-ARs are down-regulated in parallel in Pre-AA, we newly implicate sympathetic activity in inflammation-based cardiac β_1-AR desensitization.

Further in vivo and in vitro work will help determine the contribution of the sympathetic nervous system to the cardiac desensitization of cardiovascular drugs. Of particular use would be the characterization of synaptic norepinephrine turnover and sympathetic nervous system outflow. Having a solid understanding of why pharmacodynamic response in inflammation is down-regulated is critical to safely reversing the clinical problem of higher cardiovascular mortality in rheumatoid arthritis patients.

We speculate that cardiovascular pharmacodynamic desensitization contributes to exaggerated cardiovascular risk in rheumatoid arthritis patients. Continued research should investigate the changes that inflammation exerts on other systems than the immune system, such as the nervous system and its regulation of the heart. Attempts to normalize cardiovascular response should therefore encompass novel approaches that treat inflammation itself and the physiological changes that inflammation indirectly affects such as nervous system activity.

5. Appendix of miscellaneous experiments

This chapter is meant to supplement the major experimental projects that were detailed in Chapter 2 and 3. The experiments in this section are strictly preliminary, but their observations are important in their own right. As such, it is hoped that by presenting this information, this thesis will serve as a more complete reference for future work.

Sections 5.1 to 5.3 of this chapter are important lead-up experiments that were used in the selection process for determining which drug and which inflammation model would be used. The experiment in Section 5.4 was inspired by the finding that β_1-AR and norepinephrine transporter density are intimately correlated.

5.1. *Intraperitoneal atenolol dose-response curve*

Inflammatory conditions reduce cardiac responsiveness. A model of inflammation and a drug with which to test responsiveness was needed. The first step was to choose a β-blocker. We initially chose atenolol because of its β_1-AR selectivity and because it is known to be primarily excreted via the kidneys.

5.1.1. Objectives

A. To construct a dose-response curve for intra-peritoneal atenolol using PR interval as the response measurement.

B. To choose an appropriate atenolol dosage; a dose that would not be in the maximal range of response but rather would be sensitive to changes that inflammation might impose.

5.1.2. Methods

Three healthy male Sprague Dawley rats were randomly allocated into each of 5 groups: 0, 1, 5, 10, and 15 mg/kg of intraperitoneal atenolol. The rats were anesthetized and Teflon-coated electrocardiograph electrodes were implanted subcutaneously into the left and right axial regions. The next day, baseline PR interval was recorded using Acknowledge 3.01 data acquisition system (BIOPAC Systems Inc. Santa Barbara, CA). Subsequently, intraperitoneal atenolol (in sterile saline) was injected and PR intervals were recorded at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, and 6 hr. From the start of the P wave to the beginning of QRS complex was considered the PR interval. Six consecutive heart beats at each time point recorded to ensure statistical rigor.

5.1.3. Results

Figure 5-1 shows the effect of increasing atenolol doses on PR interval changes in rats. Of all the time points of all the doses, only the 4 hour mean observation for the 1 and 15 mg/kg were significantly different from zero, as analyzed by t-test.

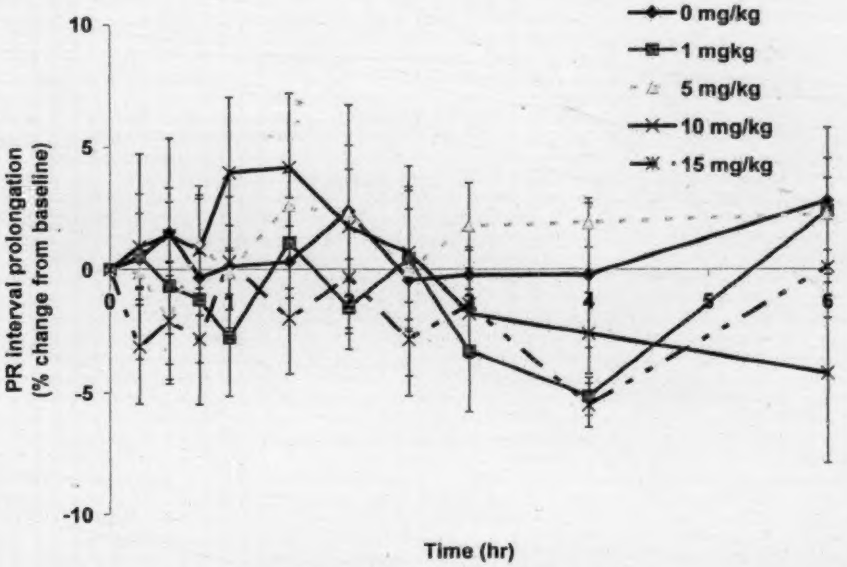

Figure 5-1. PR interval prolongation changes observed in rats injected with varying doses of intra-peritoneal atenolol versus time. n= 3, mean±SEM.

5.1.4. Conclusion

We did not find evidence that intraperitoneal atenolol prolonged PR interval in rats, even though evidence exists in human.[356] It was decided to use an alternate β-blocker.

5.2. *Intra-peritoneal β-blocker trial*

From experience, we know that propranolol consistently prolongs PR interval.[114] Before settling on propranolol as a probe for cardiac responsiveness, we wanted to conduct a trial of various β-blockers, looking at their overall potential for prolonging PR interval using our setup. We used the β-blockers listed in Table 5-1, and recognizing certain characteristics.[106]

Table 5-1. Receptor specificity and elimination characteristic of a variety of β-blockers.[106]

β-Blocker	Receptor specificity	Elimination
Metoprolol	β1/β2	Mostly hepatic
Propranolol	β1/β2	Mostly hepatic
Nadolol	β1/β2	Hepatic and renal
Sotalol	β1/β2 and Potassium channel	Renal

5.2.1. Objectives

A. To compare, using rat per group, the effect of intraperitoneal metoprolol, propranolol, nadolol, and sotalol on PR interval in the rat.

B. To introduce electrocardiogram file blinding to minimize measurement bias.

5.2.2. Methods

One rat was randomly allocated into each of 5 groups: placebo (sterile saline), metoprolol 30 mg/kg, propranolol 30 mg/kg, nadolol 40 mg/kg, and sotalol 40 mg/kg. The rats were anesthetized and Teflon-coated electrocardiograph electrodes were implanted subcutaneously into the left and right axial regions. On the next day, baseline PR intervals were recorded using Acknowledge 3.01 data acquisition system (BIOPAC Systems Inc. Santa Barbara, CA). Subsequently, the drug (in sterile saline) was injected intra-peritoneally and PR intervals were recorded at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, and 6 hr. From the start of the P wave to the beginning of QRS complex was considered the PR interval. At each recording time, 6 consecutive heart beats were measured for statistical assessment, then the files were blinded to the assessor.

5.2.3. Results

Figure 5-2 shows the AUEC for PR interval for the tested β-blockers. The rat exposed to propranolol had the highest AUEC.

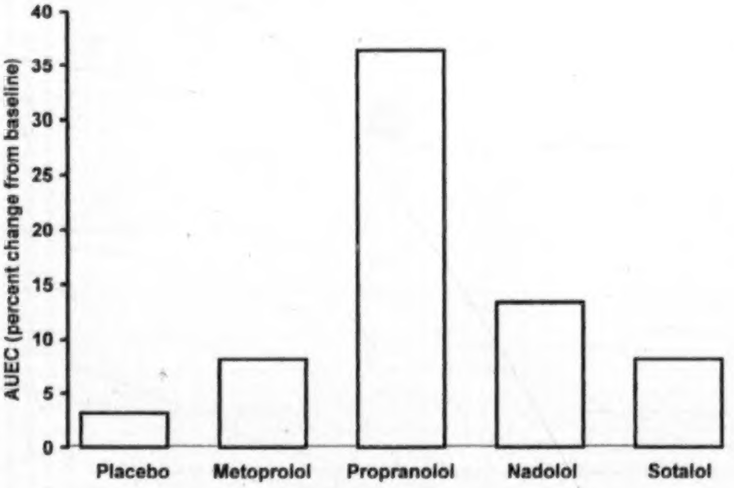

Figure 5-2. Area under the effect curve (PR-interval response) in rats injected intra-peritoneally with different β-blockers. (only one rat was used in each group)

5.2.4. Conclusion

Though not structured to provide statistical information, this brief experiment guided us to stay with propranolol as a probe for PR interval responsiveness. From a statistical perspective, the higher the effect size the lower the sample size, thereby minimizing animal use. PR interval is difficult to measure and is somewhat subjective. Blinding of the electrocardiogram files was simple because it only required a third party to rename the files, and from this point on, it became standard practice.

5.3. *Propranolol in lipopolysaccharide-induced inflammation*

Now that the decision was made to use propranolol as the cardiac responsiveness probe, we needed to decide which inflammatory model to use. Our ultimate goal was to test if statins could reverse the effects of inflammation on cardiac response. Previously, IFN-α_{2a}-based acute inflammation had been shown to reduce verapamil[327] and sotalol[125] response in rats. Administration of IFN-α_{2a} is simple and should have result in low variability of the response.

5.3.1. Hypothesis

Lipopolysaccharide-induced inflammation creates a favorable inflammation profile for testing pravastatin's anti-inflammatory effects.

5.3.2. Objective

To assess the duration of action and intensity of inflammatory effects proceeding lipopolysaccharide injection, using serum NO_X^- as a marker of inflammation.

5.3.3. Methods

Two rats were randomly allocated into each of 2 groups: placebo (sterile saline) and lipopolysaccharide. The rats were anesthetized and Teflon-coated electrocardiograph electrodes were implanted subcutaneously into the left and right axial regions, while cannulas were placed into the left jugular vein. Lipopolysaccharide or placebo (in sterile saline) was injected intraperitoneally at 8:00 AM and 5:00 PM the next day (Day 2). Blood samples were taken starting at 7:55 AM on Day 2 (just before the first injection), then every 3 hours from 12:00pm to 9:00pm, then again on Day 3, every 3 hours, from 9:00 AM until 6:00 PM, then again on Day 4, every 3 hours, from 9:00 AM until 6:00 PM. Plasma samples were analyzed for NO_X^- using a modified Griess reaction as described in Chapter 2.

5.3.4. Results

Figure 5-3 shows the effects of lipopolysaccharide injection on the inflammatory profile of plasma nitrite in rats. The twice daily injections were apparently insufficient for causing 24 hours of inflammatory changes.

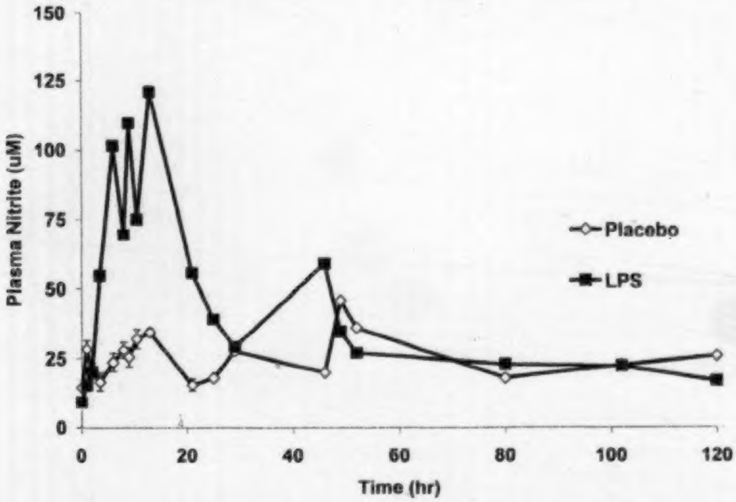

Figure 5-3. Plasma NO_X^- in male Sprague-Dawley rats treated with intraperitoneal injections of 1 mg/kg lipopolysaccharide dosed at 8:00 AM and 5:00 PM (n=2).

5.3.5. Conclusion

We found that inflammation associated with lipopolysaccharide was not likely to provide day to day consistency in inflammation, and therefore not considered favorable for testing the anti-inflammatory effects of statins. These results prompted us to choose the Pre-AA model for our main experiment. This model would offer smoother inflammatory mediator profiles and less possibility for fluctuations in inflammatory mediators during the propranolol time course study.

5.4. *6-Hydroxydopamine sympatholysis in adjuvant arthritis*

In Chapter 3 we showed that inflammatory mediators are weak predictors of pharmacodynamic response. The strongest correlation for any of our measured proteins and/or inflammatory mediators was between norepinephrine transporter density and β_1-AR density. In contrast, there was no correlation between inflammatory mediators and β_1-AR density.

Post-β_1-AR signal transduction pathways were assessed by measuring G-protein-coupled receptor kinase-2 and L-type calcium channels. With the measurement of norepinephrine transporter we have begun to examine pre-receptor alterations in sympathetic signaling.

In this study we have used 6-hydroxydopamine, as a neurotoxin, to knock out the peripheral sympathetic nervous system. Hence, 6-hydroxydopamine reduces norepinephrine signaling.[357] 6-Hydroxydopamine sympatholysis has been used in a variety of different animal studies. Table 5-2 details some of these studies and was useful in establishing a protocol for 6-hydroxydopamine administration in this lab. Confirmation of successful neurolysis can be achieved by using tyramine sensitivity. Tyramine normally causes the rapid release of norepinephrine from the nerve terminals. 6-hydroxydopamine animals should have reductions in tyramine response in the order of 80-95% (as assessed by blood pressure.

Table 5-2. 6-Hydroxydopamine dosing schemes used in a variety rat disease models. Cardioprotectants are often given with the first 6-hydroxydopamine dose to prevent sympathetic overload which can cause damage to the heart. A reduction in tyramine can be used as a control to confirm successful sympatholysis. Abbreviations: CHF=congestive heart failure, Freq.=frequency.

Author	Purpose of Study	Dose (mg/kg)	Freq.	Route	Duration	Cardioprotectants	Tyramine Response
Perlini et al [358]	Congestive heart failure	150	2/wk	i.p.	10 wk	None	Not done
Kompa et al[359]	Beta-receptor changes with isoprenaline	100	1/wk	i.v.	1 wk		↓ - 94% (BP)
Marano et al [360]	Nicotine vasoactivity	100	2-3/wk	i.p., i.p., i.v.	1 wk	phentolamine with first dose	↓ - 90% (BP) ↓ - 90% (HR)
Ferrari et al [361]	Baroreceptor-heart rate reflex	100-150	2-3/wk	i.p., i.p., i.v.	1 wk	phentolamine with first dose	↓ - 80% (BP) ↓ - 72% (HR)
Kimet al [362]	Dermal wound healing	50,50, 100, 100, 100,100	irregular	i.p.	21 d	none	Not done
Hansson et al [363]	Natriuretic peptide levels	100	daily	s.c.	3 d	none	Not done
Mircoliet al [364]	Baroreflex in CHF	100	2/wk	i.p.	5 wk	none	Not done
Sterin-Borda et al [365]	IL-2 effects on atria	16.5	once	i.v.	once	none	refractory
Carson et al [357]	Neuropathic tachycardia syndrome	150	once	i.v.	once	propranolol, prazosin	↓ - 88% (BP)

Activation of the sympathetic nervous system can lead to a state of chronic agonism at the β_1-AR. In turn, downregulation of the β_1-AR may occur. By knocking out the peripheral sympathetic nervous system we can protect the β_1-AR from the stimulatory effects of a hyperactive sympathetic nervous system. This could make it possible to quantify the relative contribution of direct inflammatory effects (at the cardiomyocyte) to indirect inflammatory effects (by altering the sympathetic nervous system).

5.4.1. Hypothesis

That 6-hydroxydopamine-induced sympatholysis, in the adjuvant arthritis rat model, will help preserve β_1-AR responsiveness to isoproterenol by reducing cardiac sympathetic activity.

5.4.2. Objectives

A. To determine isoproterenol response in 6-hydroxydopamine animals compared to placebo- and atorvastatin-treated adjuvant arthritis rats using heart rate.
B. To assess the ratio of the sympathetic to parasympathetic nerve activity in these animals using a power spectrum analysis of heart rate variability.
C. To verify sympatholytic success using tyramine response as measured by PR interval.

Methods:

Two rats were randomly allocated into each of three groups as follows:

	Complete Freund's adjuvant	Placebo or atorvastatin 5mg/kg twice daily	6-OHDA*	Cardioprotective solution (Propranolol 10mg/kg, Prazosin 2mg/kg)
6-OHDA group	Yes	placebo	Yes	Days 0 and 3
Placebo group	Yes	placebo	No	Days 0 and 3
Atorvastatin group	Yes	atorvastatin	No	Days 0 and 3

* 6-OHDA = 6-hydroxydopamine

On Day 0 all groups received *Mycobacterium butyricum* in squalene as a Complete Freund's adjuvant. Since the study was finished by Day 11 no clinical symptoms of inflammation should be expected. On Days 0, 3, 6 and 9, rats were deeply anesthetized using a halothane and oxygen mixture, followed by an intraperitoneal injection of 148 mg/kg of 6-hydroxydopamine hydrobromide or placebo (vehicle, 0.1% ascorbic acid in normal saline). This injection was administered with propranolol and prazosin to protect the heart from sympathetic overload, which can occur on the first and second doses of hydroxydopamine. Atorvastatin 5 mg/kg twice daily or placebo (1% methylcellulose in water) was administered on Days 0 through 11. On Day 11 the rats were anesthetized and Teflon-coated electrocardiograph electrodes were implanted subcutaneously into the left and right axial regions, while cannulas were placed into the left jugular vein. The rats rested for four hours.

Power spectrum analysis was performed by analysis of heart rate variability, using a 5 minute continuous electrocardiographic recording. The relative amount of sympathetic nervous system activity to parasympathetic nervous system activity was taken to be the integral power (ms^2) of the low frequency (sympathetic and parasympathetic influence) divided by the integral power of the high frequency (parasympathetic influence). Low frequency was taken to be in the 0.25-0.6 Hz range, and high frequency was taken to be in the 1.0-2.0 Hz range.

For intravenous isoproterenol response (using 0.1856 μg/ml in sterile saline) on Day 11, the animals had 12 min continuous electrocardiogram recordings for assessing heart rate response. After recording for 1 minute, isoproterenol was infused at 0.05 μg/kg over 30 seconds. At 7 minutes the isoproterenol was infused at 0.25 μg/kg over 30 seconds. Heart rate was used to assess isoproterenol response by measuring the maximum heart rate change that occurred during the infusion.

For intravenous tyramine response (using 0.406 mg/ml in sterile saline) was performed 2 hours after isoproterenol testing. It followed the same pattern but with different doses. After recording the electrocardiogram for 1 minute, tyramine was infused at 0.05 μg/kg over 30 seconds, then again at 7 minutes at 0.25 μg/kg over 30 seconds. The recording was stopped at 12 minutes. The PR interval was used to assess responsiveness to tyramine because the animals did not have their blood pressure monitored. Analysis of PR interval was performed under blinded conditions. Measurements were made on the electrocardiogram corresponding to the midpoint of either the isoproterenol or tyramine infusion as appropriate.

Heart rate, heart rate variability, and power spectrum analysis was analyzed using Acknowledge software version 3.9.1 (Biopac Systems Inc.).

5.4.3. Results

Figure 5-4 shows that 0.25 μg/kg isoproterenol infusion increased average heart rate, and that the placebo-treated animals had the smallest increase in heart rate. Similar findings were observed with the 0.05 μg/kg infusion (data not shown). One animal was excluded from the isoproterenol graph because inspection of the animal's ECG revealed heart damage which may have occurred during the flushing of the cannula. (Figure 5-4).

As expected, animals in the 6-hydroxydopamine group had the smallest sympathetic nervous system activity (Figure 5-5). We confirmed successful sympatholysis using PR interval response to 0.5 mg/kg tyramine infusion (Figure 5-6). The lower dose tyramine infusion was not analyzed.

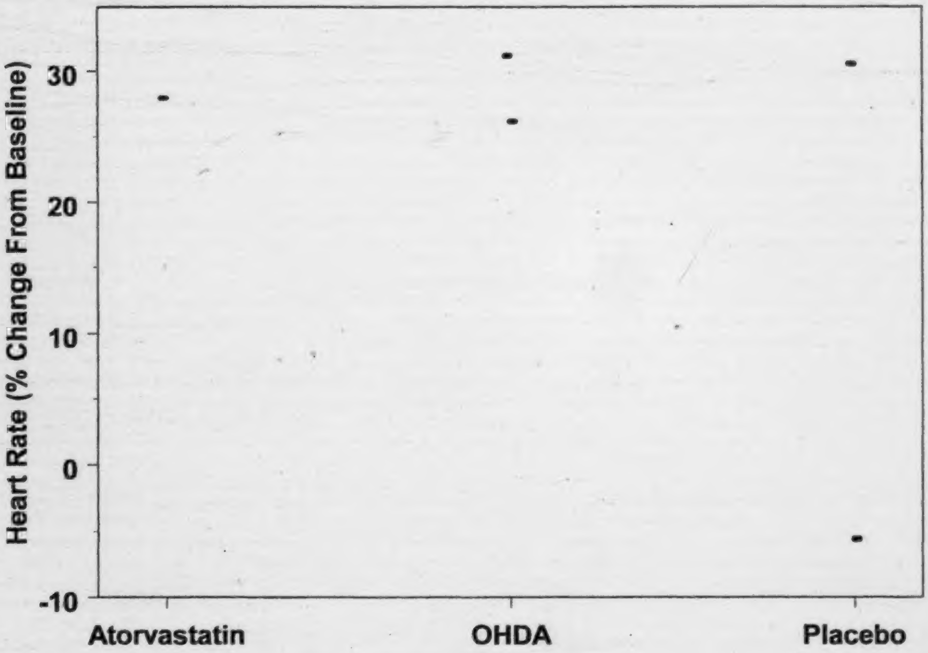

Figure 5-4. Isoproterenol response (changes in heart rate) in rats treated that have been treated with atorvastatin or sympatholysis (OHDA). Each point represents one animal, n=1-2.

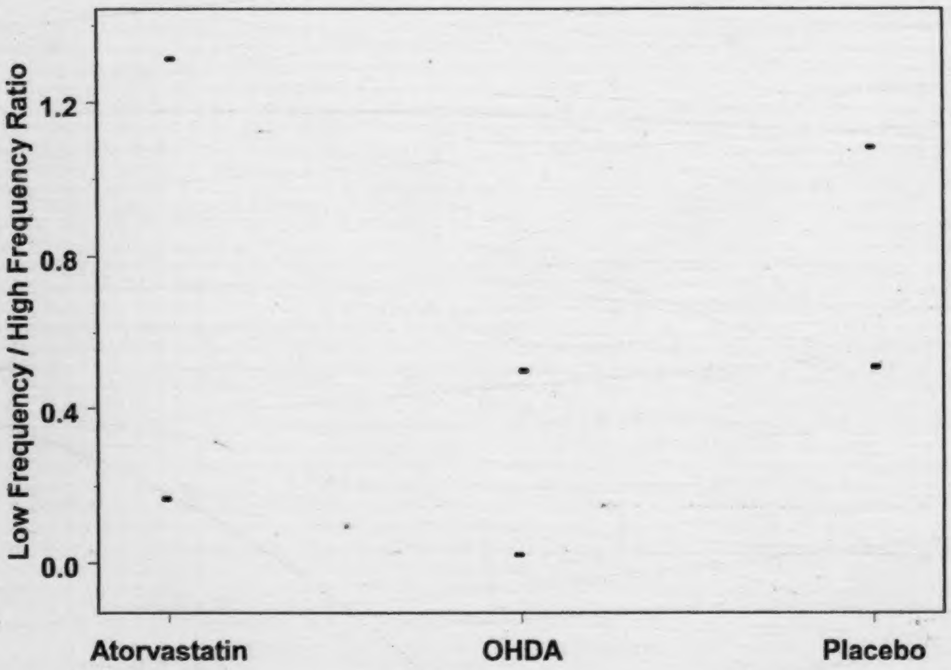

Figure 5-5. Cardiac sympathetic nervous system activity (as measured by the low to high frequency power ratio) in rats treated that have been treated with atorvastatin or sympatholysis (OHDA). Each point represents one animal, n=2.

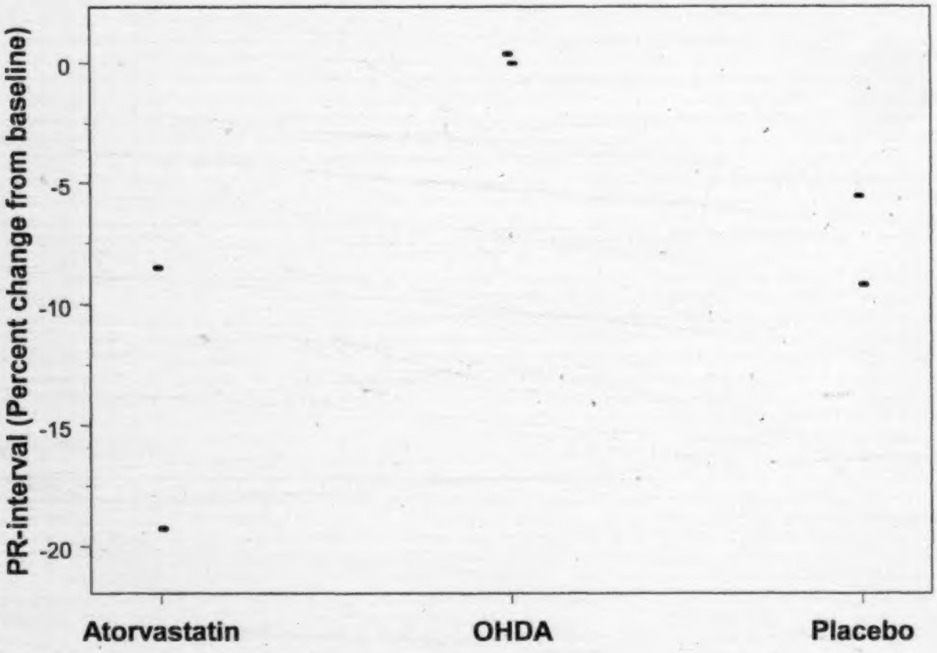

Figure 5-6. Tyramine response (PR interval response) in rats treated that have been treated with atorvastatin or sympatholysis (OHDA) confirms that the rats have been successfully sympatholysed. PR interval is expected to be shortened in the atorvastatin and placebo groups because tyramine stimulates norepinephrine release. Each point represents one animal, n=2.

5.4.4. Conclusion

β-AR responsiveness to isoproterenol may be reduced in inflamed animals. Sympatholysis or atorvastatin administration may protect the loss of β-AR response (Figure 5-4). This suggests that inflammation's effects on the nervous system contribute to reduced pharmacodynamic response found with cardiovascular drugs. Power spectrum analysis suggests that sympathetic activity is reduced in the 6-hydroxydopamine group (Figure 5-5), as does reduced tyramine response (Figure 5-6).

This preliminary study is limited by the inability to perform statistics and its small sample size. Nevertheless, it does justify performing further experiments to confirm these findings, and also helps to establish these procedures in the lab.

6. References

1. **Szekanecz Z, Kerekes G, Der H, Sandor Z, Szabo Z, Vegvari A, Simkovics E, Soos L, Szentpetery A, Besenyei T, Szucs G, Szanto S, Tamasi L, et al.** Accelerated Atherosclerosis in Rheumatoid Arthritis. *Ann N Y Acad Sci.* 2007;1108:349-58.
2. **Houston MC.** Nonsteroidal Anti-Inflammatory Drugs and Antihypertensives. *Am J Med.* 1991;90(5A):42S-47S.
3. **Verheggen PW, de Maat MP, Cats VM, Haverkate F, Zwinderman AH, Kluft C, Bruschke AV.** Inflammatory Status as a Main Determinant of Outcome in Patients with Unstable Angina, Independent of Coagulation Activation and Endothelial Cell Function. *Eur Heart J.* 1999;20(8):567-74.
4. **Mayo PR, Skeith K, Russell AS, Jamali F.** Decreased Dromotropic Response to Verapamil Despite Pronounced Increased Drug Concentration in Rheumatoid Arthritis. *Br J Clin Pharmacol.* 2000;50(6):605-613.
5. **Endres M.** Statins: Potential New Indications in Inflammatory Conditions. *Atherosclerosis Supplements.* 2006;7(1):31-35.
6. **Kulmatycki KM, Jamali F.** Therapeutic Relevance of Altered Cytokine Expression. *Cytokine.* 2001;14(1):1-10.
7. **Kuby J**, *Immunology.* 1997, New York: WH Freeman and Company.
8. **Schmid-Schonbein GW.** Analysis of Inflammation. *Annu Rev Biomed Eng.* 2006;8:93-131.

9. **Kidd P.** Th1/Th2 Balance: The Hypothesis, Its Limitations, and Implications for Health and Disease. *Altern Med Rev.* 2003;8(3):223-46.

10. **Parslow TG, Stites DP, Terr AI, Imboden JB**, *Medical Immunology.* 10 ed. 2001: The McGraw-Hill Companies, Inc.

11. **McPhee SJ, Papadakis MA, L.M. T, Gonzales R, Zeiger R**, *Current Medical Diagnosis & Treatment.* 47 ed. 2008: The McGraw-Hill Companies, Inc.

12. **Dagenais NJ, Jamali F.** Protective Effects of Angiotensin II Interruption: Evidence for Antiinflammatory Actions. *Pharmacotherapy.* 2005;25(9):1213-29.

13. **Billiau A, Matthys P.** Modes of Action of Freund's Adjuvants in Experimental Models of Autoimmune Diseases. *J Leukoc Biol.* 2001;70(6):849-60.

14. **Schulze-Koops H, Kalden JR.** The Balance of Th1/Th2 Cytokines in Rheumatoid Arthritis. *Best Practice & Research Clinical Rheumatology.* 2001;15(5):677-691.

15. **Abbas AK, Murphy KM, Sher A.** Functional Diversity of Helper T Lymphocytes. *Nature.* 1996;383(6603):787-93.

16. **Hakamada-Taguchi R, Uehara Y, Kuribayashi K, Numabe A, Saito K, Negoro H, Fujita T, Toyo-oka T, Kato T.** Inhibition of Hydroxymethylglutaryl-Coenzyme a Reductase Reduces Th1 Development and Promotes Th2 Development. *Circ Res.* 2003;93(10):948-956.

17. **Constant SL, Bottomly K.** Induction of Th1 and Th2 CD$_4^+$ T Cell Responses: The Alternative Approaches. *Annual Review of Immunology.* 1997;15(1):297-322.

18. **Uyemura K, Demer LL, Castle SC, Jullien D, Berliner JA, Gately MK, Warrier RR, Pham N, Fogelman AM, Modlin RL.** Cross-Regulatory Roles of Interleukin (IL)-12 and IL-10 in Atherosclerosis. *J. Clin. Invest.* 1996;97(9):2130-2138.

19. **Barnes PJ, Chung KF, Page CP.** Inflammatory Mediators of Asthma: An Update. *Pharmacol Rev.* 1998;50(4):515-596.

20. **Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, van den Berg WB.** Role of Interleukin-4 and Interleukin-10 in Murine Collagen-Induced Arthritis. Protective Effect of Interleukin-4 and Interleukin-10 Treatment on Cartilage Destruction. *Arthritis Rheum.* 1997;40(2):249-60.

21. **Romagnani S.** Th1 and Th2 in Human Diseases. *Clinical Immunology and Immunopathology.* 1996;80(3):225-235.

22. **Romagnani P, Annunziato F, Piccinni MP, Maggi E, Romagnani S.** Th1/Th2 Cells, Their Associated Molecules and Role in Pathophysiology. *Eur Cytokine Netw.* 2000;11(3):510-1.

23. **Gerli R, Goodson NJ.** Cardiovascular Involvement in Rheumatoid Arthritis. *Lupus.* 2005;14(9):679-82.

24. **Libby P, Ridker PM, Maseri A.** Inflammation and Atherosclerosis. *Circulation.* 2002;105(9):1135-43.

25. **Hayden MS, West AP, Ghosh S.** Nf-Kappa-B and the Immune Response. *Oncogene.*25(51):6758-6780.

26. **Kranzhöfer R, Browatzki M, Schmidt J, Kubler W.** Angiotensin Ii Activates the Proinflammatory Transcription Factor Nuclear Factor-Kappa-B in Human Monocytes. *Biochem Biophys Res Commun.* 1999;257(3):826-8.

27. **Schmidt AM, Yan SD, Wautier JL, Stern D.** Activation of Receptor for Advanced Glycation End Products: A Mechanism for Chronic Vascular Dysfunction in Diabetic Vasculopathy and Atherosclerosis. *Circ Res.* 1999;84(5):489-97.

28. **Davi G, Falco A.** Oxidant Stress, Inflammation and Atherogenesis. *Lupus.* 2005;14(9):760-4.

29. **Chung CP, Avalos I, Raggi P, Stein CM.** Atherosclerosis and Inflammation: Insights from Rheumatoid Arthritis. *Clin Rheumatol.* 2007;26(8):1228-33.

30. **Rose-John S, Scheller J, Elson G, Jones SA.** Interleukin-6 Biology Is Coordinated by Membrane-Bound and Soluble Receptors: Role in Inflammation and Cancer. *J Leukoc Biol.* 2006;80(2):227-236.

31. **Aukrust P, Gullestad L, Ueland T, Damas JK, Yndestad A.** Inflammatory and Anti-Inflammatory Cytokines in Chronic Heart Failure: Potential Therapeutic Implications. *Ann Med.* 2005;37(2):74-85.

32. **Ito T, Ikeda U.** Inflammatory Cytokines and Cardiovascular Disease. *Curr Drug Targets Inflamm Allergy.* 2003;2(3):257-65.

33. **Farzaneh-Far A, Rudd J, Weissberg PL.** Inflammatory Mechanisms: Ischaemic Heart Disease. *Br Med Bull.* 2001;59(1):55-68.

34. **Tesfamariam B, DeFelice AF.** Endothelial Injury in the Initiation and Progression of Vascular Disorders. *Vascular Pharmacology.* 2007;46(4):229-237.

35. **Ross R.** Atherosclerosis -- an Inflammatory Disease. *N Engl J Med.* 1999;340(2):115-126.

36. **Rasmussen LM, Hansen PR, Nabipour MT, Olesen P, Kristiansen MT, Ledet T.** Diverse Effects of Inhibition of 3-Hydroxy-3-Methylglutaryl-CoA Reductase on the Expression of VCAM-1 and E-Selectin in Endothelial Cells. *Biochem J.* 2001;360(Pt 2):363-70.

37. **Marx N, Sukhova GK, Collins T, Libby P, Plutzky J.** PPAR-Alpha Activators Inhibit Cytokine-Induced Vascular Cell Adhesion Molecule-1 Expression in Human Endothelial Cells. *Circulation.* 1999;99(24):3125-3131.

38. **Napoli C, Ignarro LJ.** Nitric Oxide and Atherosclerosis. *Nitric Oxide.* 2001;5(2):88-97.

39. **Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK.** Regional Accumulations of T Cells, Macrophages, and Smooth Muscle Cells in the Human Atherosclerotic Plaque. *Arteriosclerosis.* 1986;6(2):131-8.

40. **Plutzky J.** Inflammatory Pathways in Atherosclerosis and Acute Coronary Syndromes. *The American Journal of Cardiology.* 2001;88(8, Supplement 1):10-15.

41. **Yamashita T, Freigang S, Eberle C, Pattison J, Gupta S, Napoli C, Palinski W.** Maternal Immunization Programs Postnatal Immune Responses and Reduces Atherosclerosis in Offspring. *Circ Res.* 2006;99(7):E51-64.

42. **Bobryshev YV.** Monocyte Recruitment and Foam Cell Formation in Atherosclerosis. *Micron.* 2006;37(3):208-22.

43. **Sariahmetoglu M, Crawford BD, Leon H, Sawicka J, Li L, Ballermann BJ, Holmes C, Berthiaume LG, Holt A, Sawicki G, Schulz R.** Regulation of Matrix Metalloproteinase-2 (MMP-2) Activity by Phosphorylation. *Faseb J.* 2007;21(10):2486-95.

44. **Liu P, Sun M, Sader S.** Matrix Metalloproteinases in Cardiovascular Disease. *Can J Cardiol.* 2006;22 Suppl B:25B-30B.

45. **Wang W, Sawicki G, Schulz R.** Peroxynitrite-Induced Myocardial Injury Is Mediated through Matrix Metalloproteinase-2. *Cardiovascular Research.* 2002;53(1):165-174.

46. **Li YY, Feldman AM, Sun Y, McTiernan CF.** Differential Expression of Tissue Inhibitors of Metalloproteinases in the Failing Human Heart. *Circulation.* 1998;98(17):1728-1734.

47. **Gao CQ, Sawicki G, Suarez-Pinzon WL, Csont T, Wozniak M, Ferdinandy P, Schulz R.** Matrix Metalloproteinase-2 Mediates Cytokine-Induced Myocardial Contractile Dysfunction. *Cardiovasc Res.* 2003;57(2):426-33.

48. **Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, Szczesna-Cordary D, Schulz R.** Degradation of Myosin Light Chain in Isolated Rat Hearts Subjected to Ischemia-Reperfusion Injury: A New Intracellular Target for Matrix Metalloproteinase-2. *Circulation.* 2005;112(4):544-552.

49. **Albert MA, Staggers J, Chew P, Ridker PM.** The Pravastatin Inflammation CRP Evaluation (PRINCE): Rationale and Design. *American Heart Journal.* 2001;141(6):893-898.

50. **Ridker PM, Cannon CP, Morrow D, Rifai N, Rose LM, McCabe CH, Pfeffer MA, Braunwald E.** C-Reactive Protein Levels and Outcomes after Statin Therapy. *N Engl J Med.* 2005;352(1):20-8.

51. **Symmons DPM.** Epidemiology of Rheumatoid Arthritis: Determinants of Onset, Persistence and Outcome. *Best Practice & Research Clinical Rheumatology.* 2002;16(5):707-722.

52. **Scott DL, Smith C, Kingsley G.** What Are the Consequences of Early Rheumatoid Arthritis for the Individual? *Best Practice & Research Clinical Rheumatology.* 2005;19(1):117-136.

53. **Rheumatology ACo.** Guidelines for the Management of Rheumatoid Arthritis: 2002 Update. *Arthritis Rheum.* 2002;46(2):328-346.

54. **Ollier WER, Harrison B, Symmons D.** What Is the Natural History of Rheumatoid Arthritis? *Best Practice & Research Clinical Rheumatology.* 2001;15(1):27-48.

55. **Haroon N, Aggarwal A, Lawrence A, Agarwal V, Misra R.** Impact of Rheumatoid Arthritis on Quality of Life. *Modern Rheumatology.* 2007;17(4):290-295.

56. **Alamanos Y, Drosos AA.** Epidemiology of Adult Rheumatoid Arthritis. *Autoimmunity Reviews.* 2005;4(3):130-136.

57. **Oliver JE, Worthington J, Silman AJ.** Genetic Epidemiology of Rheumatoid Arthritis. *Curr Opin Rheumatol.* 2006;18(2):141-6.

58. **Carroll L, Hannawi S, Marwick T, Thomas R.** Rheumatoid Arthritis: Links with Cardiovascular Disease and the Receptor for Advanced Glycation End Products. *Wien Med Wochenschr.* 2006;156(1-2):42-52.

59. **Maradit-Kremers H, Nicola PJ, Crowson CS, Ballman KV, Gabriel SE.** Cardiovascular Death in Rheumatoid Arthritis: A Population-Based Study. *Arthritis Rheum.* 2005;52(3):722-32.

60. **Snow MH, Mikuls TR.** Rheumatoid Arthritis and Cardiovascular Disease: The Role of Systemic Inflammation and Evolving Strategies of Prevention. *Curr Opin Rheumatol.* 2005;17(3):234-41.

61. **Klocke R, Cockcroft JR, Taylor GJ, Hall IR, Blake DR.** Arterial Stiffness and Central Blood Pressure, as Determined by Pulse Wave Analysis, in Rheumatoid Arthritis. *Ann Rheum Dis.* 2003;62(5):414-418.

62. **Gabriel S.** Heart Disease in Rheumatoid Arthritis: Changing the Paradigm of Systemic Inflammatory Disorders. *J Rheumatol.* 2007;34(1):220-3.

63. **Solomon DH, Karlson EW, Rimm EB, Cannuscio CC, Mandl LA, Manson JE, Stampfer MJ, Curhan GC.** Cardiovascular Morbidity and

Mortality in Women Diagnosed with Rheumatoid Arthritis. *Circulation*. 2003;107(9):1303-7.

64. **Maradit-Kremers H, Crowson CS, Nicola PJ, Ballman KV, Roger VL, Jacobsen SJ, Gabriel SE.** Increased Unrecognized Coronary Heart Disease and Sudden Deaths in Rheumatoid Arthritis: A Population-Based Cohort Study. *Arthritis Rheum*. 2005;52(2):402-11.

65. **Scrivo R, Di Franco M, Spadaro A, Valesini G.** The Immunology of Rheumatoid Arthritis. 2007;1108(1):312-322.

66. **Walsh NC, Crotti TN, Goldring SR, Gravallese EM.** Rheumatic Diseases: The Effects of Inflammation on Bone. *Immunol Rev*. 2005;208(1):228-251.

67. **Gaffo A, Saag KG, Curtis JR.** Treatment of Rheumatoid Arthritis. *Am J Health Syst Pharm*. 2006;63(24):2451-65.

68. **Sattar N, McCarey DW, Capell H, McInnes IB.** Explaining How "High-Grade" Systemic Inflammation Accelerates Vascular Risk in Rheumatoid Arthritis. *Circulation*. 2003;108(24):2957-2963.

69. **Dekkers JC, Geenen R, Godaert GL, Bijlsma JW, van Doornen LJ.** Elevated Sympathetic Nervous System Activity in Patients with Recently Diagnosed Rheumatoid Arthritis with Active Disease. *Clin Exp Rheumatol*. 2004;22(1):63-70.

70. **Gonzalez-Gay MA, Gonzalez-Juanatey C, Miranda-Filloy JA, Garcia-Porrua C, Llorca J, Martin J.** Cardiovascular Disease in Rheumatoid Arthritis. *Biomedecine & Pharmacotherapy*. 2006;60(10):673-677.

71. **Crea F, Biasucci LM, Buffon A, Liuzzo G, Monaco C, Caligiuri G, Kol A, Sperti G, Cianflone D, Maseri A.** Role of Inflammation in the Pathogenesis of Unstable Coronary Artery Disease. *Am J Cardiol.* 1997;80(5A):10E-16E.

72. **Prior P, Symmons DP, Scott DL, Brown R, Hawkins CF.** Cause of Death in Rheumatoid Arthritis. *Br J Rheumatol.* 1984;23(2):92-9.

73. **Giles J, Fernandes V, Lima J, Bathon J.** Myocardial Dysfunction in Rheumatoid Arthritis: Epidemiology and Pathogenesis. *Arthritis Res Ther.* 2005;7(5):195 - 207.

74. **Ridker PM, Rifai N, Stampfer MJ, Hennekens CH.** Plasma Concentration of Interleukin-6 and the Risk of Future Myocardial Infarction among Apparently Healthy Men. *Circulation.* 2000;101(15):1767-72.

75. **Blake GJ, Ridker PM.** Novel Clinical Markers of Vascular Wall Inflammation. *Circ Res.* 2001;89(9):763-771.

76. **Evrengül H, Dursunoglu D, Cobankara V, Polat BI, Seleci D, Kabukçu S, Kaftan A, Semiz E, Kilic M.** Heart Rate Variability in Patients with Rheumatoid Arthritis. *Rheumatology International.* 2004;24(4):198-202.

77. **White WB.** Cardiovascular Effects of the Cyclooxygenase Inhibitors. *Hypertension.* 2007;49(3):408-18.

78. **Joe B, Griffiths MM, Remmers EF, Wilder RL.** Animal Models of Rheumatoid Arthritis and Related Inflammation. *Curr Rheumatol Rep.* 1999;1(2):139-48.

79. **Ling S, Jamali F.** Effect of Early Phase Adjuvant Arthritis on Hepatic P450 Enzymes and Pharmacokinetics of Verapamil: An Alternative Approach to the Use of an Animal Model of Inflammation for Pharmacokinetic Studies. *Drug Metab Dispos.* 2005;33(4):579-86. Epub 2005 Jan 19.

80. **Dessein P, Joffe B, Singh S.** Biomarkers of Endothelial Dysfunction, Cardiovascular Risk Factors and Atherosclerosis in Rheumatoid Arthritis. 2005;7(3):R634 - R643.

81. **Sigidin YA, Loukina GV, Skurkovich B, Skurkovich S.** Randomized, Double-Blind Trial of Anti-Interferon-Gamma Antibodies in Rheumatoid Arthritis. *Scand J Rheumatol.* 2001;30(4):203-7.

82. **Clements JD, Jamali F.** Pravastatin Reverses the Down-Regulating Effect of Inflammation on B-Adrenergic Receptors: A Disease-Drug Interaction between Inflammation, Pravastatin, and Propranolol. *Vascular Pharmacology.* 2007;46(1):52-59.

83. **Hanafy S, Dagenais NJ, Dryden WF, Jamali F.** Effects of Angiotensin II Blockade on Inflammation-Induced Alterations of Pharmacokinetics and Pharmacodynamics of Calcium Channel Blockers. *Br J Pharmacol.* 2007.

84. **Kimura T, Higaki K.** Gastrointestinal Transit and Drug Absorption. *Biol Pharm Bull.* 2002;25(2):149-64.

85. **Shargel L, Wu-Pong S, Yu ABC**, *Applied Biopharmaceutics and Pharmacokinetics*. 5th ed. 2005: McGraw-Hill.

86. **Davies NM, Wright MR, Jamali F.** Antiinflammatory Drug-Induced Small Intestinal Permeability: The Rat Is a Suitable Model. *Pharm Res.* 1994;11(11):1652-6.

87. **McKay DM, Baird AW.** Cytokine Regulation of Epithelial Permeability and Ion Transport. *Gut.* 1999;44(2):283-289.

88. **Farhadi A, Fields JZ, Keshavarzian A.** Mucosal Mast Cells Are Pivotal Elements in Inflammatory Bowel Disease That Connect the Dots: Stress, Intestinal Hyperpermeability and Inflammation. *World J Gastroenterol.* 2007;13(22):3027-30.

89. **Brocks DR, Skeith KJ, Johnston C, Emamibafrani J, Davis P, Russell AS, Jamali F.** Hematologic Disposition of Hydroxychloroquine Enantiomers. *J Clin Pharmacol.* 1994;34(11):1088-97.

90. **Kremer JM, Wilting J, Janssen LH.** Drug Binding to Human Alpha-1-Acid Glycoprotein in Health and Disease. *Pharmacol Rev.* 1988;40(1):1-47.

91. **Kulmatycki KM, Jamali F.** Drug Disease Interactions: Role of Inflammatory Mediators in Disease and Variability in Drug Response. *J Pharm Pharm Sci.* 2005;8(3):602-25.

92. **Benet LZ, Hoener BA.** Changes in Plasma Protein Binding Have Little Clinical Relevance. *Clin Pharmacol Ther.* 2002;71(3):115-21.

93. **Fernandez C, Buyse M, German-Fattal M, Gimenez F.** Influence of the Pro-Inflammatory Cytokines on P-Glycoprotein Expression and Functionality. *J Pharm Pharm Sci.* 2004;7(3):359-71.

94. **Pauli-Magnus C, von Richter O, Burk O, Ziegler A, Mettang T, Eichelbaum M, Fromm MF.** Characterization of the Major Metabolites of Verapamil as Substrates and Inhibitors of P-Glycoprotein. *J Pharmacol Exp Ther.* 2000;293(2):376-82.

95. **Weiss M, Kang W.** P-Glycoprotein Inhibitors Enhance Saturable Uptake of Idarubicin in Rat Heart: Pharmacokinetic/Pharmacodynamic Modeling. *J Pharmacol Exp Ther.* 2002;300(2):688-694.

96. **Seelig A, Landwojtowicz E.** Structure-Activity Relationship of P-Glycoprotein Substrates and Modifiers. *European Journal of Pharmaceutical Sciences.* 2000;12(1):31-40.

97. **Meissner K, Sperker B, Karsten C, zu Schwabedissen HM, Seeland U, Bohm M, Bien S, Dazert P, Kunert-Keil C, Vogelgesang S, Warzok R, Siegmund W, Cascorbi I, et al.** Expression and Localization of P-Glycoprotein in Human Heart: Effects of Cardiomyopathy. *J. Histochem. Cytochem.* 2002;50(10):1351-1356.

98. **Sims JJ, Neudeck BL, Loeb JM, Wiegert NA.** Tachycardia-Induced Heart Failure Does Not Alter Myocardial P-Glycoprotein Expression. *Pharmacotherapy.* 2004;24(1):1-7.

99. **Renton KW.** Alteration of Drug Biotransformation and Elimination During Infection and Inflammation. *Pharmacology & Therapeutics.* 2001;92(2-3):147-163.

100. **Iber H, Sewer MB, Barclay TB, Mitchell SR, Li T, Morgan ET.** Modulation of Drug Metabolism in Infectious and Inflammatory Diseases. *Drug Metab Rev.* 1999;31(1):29-41.

101. **Emami J, Pasutto FM, Jamali F.** Effect of Experimental Diabetes Mellitus and Arthritis on the Pharmacokinetics of Hydroxychloroquine Enantiomers in Rats. *Pharm Res.* 1998;15(6):897-903.

102. **Monshouwer M, Witkamp RF, Nijmeijer SM, Van Amsterdam JG, Van Miert ASJPAM.** Suppression of Cytochrome P450- and Udp Glucuronosyl Transferase-Dependent Enzyme Activities by Proinflammatory Cytokines and Possible Role of Nitric Oxide in Primary Cultures of Pig Hepatocytes. *Toxicology and Applied Pharmacology.* 1996;137(2):237-244.

103. **Meunier CJ, Verbeeci RK.** Glucuronidation of R- and S-Ketoprofen by Liver Microsomes of Adjuvant-Induced Arthritic Rats. *European Journal of Pharmaceutical Sciences.* 1996;4(Supplement 1):S98.

104. **Berne RM, Levy MN**, *Cardiovascular Physiology*. 8 ed. 2001, St. Louis: Mosby.

105. **Klabunde RE**. PR-Interval Graph. [Webpage] 2007 [cited 2007 December 20]; Available from: http://www.cvphysiology.com/ Arrhythmias/ECG%20trace%20with%20grid.gif.

106. **American Society of Health-System Pharmacists**. AHFS Drug Information. 2007 [cited 2007 Dec 3].

107. **Shand DG.** Pharmacokinetics of Propranolol: A Review. *Postgrad Med J.* 1976;52 Suppl 4:22-25.

108. **Schneider RE, Babb J, Bishop H, Mitchard M, Hoare AM.** Plasma Levels of Propranolol in Treated Patients with Coeliac Disease and Patients with Crohn's Disease. *Br Med J.* 1976;2(6039):794-5.

109. **Schneider RE, Bishop H, Hawkins CF.** Plasma Propranolol Concentrations and the Erythrocyte Sedimentation Rate. *Br J Clin Pharmacol.* 1979;8(1):43-7.

110. **Schneider RE, Bishop H, Kendall MJ, Quarterman CP.** Effect of Inflammatory Disease on Plasma Concentrations of Three Beta-Adrenoceptor Blocking Agents. *Int J Clin Pharmacol Ther Toxicol.* 1981;19(4):158-62.

111. **Piafsky KM, Borga O, Odar-Cederlof I, Johansson C, Sjoqvist F.** Increased Plasma Protein Binding of Propranolol and Chlorpromazine Mediated by Disease-Induced Elevations of Plasma Alpha1 Acid Glycoprotein. *N Engl J Med.* 1978;299(26):1435-9.

112. **Walker KA, Barber HE, Hawksworth GM.** Mechanism Responsible for Altered Propranolol Disposition in Adjuvant-Induced Arthritis in the Rat. *Drug Metab Dispos.* 1986;14(4):482-6.

113. **Piquette-Miller M, Jamali F.** Selective Effect of Adjuvant Arthritis on the Disposition of Propranolol Enantiomers in Rats Detected Using a Stereospecific HPLC Assay. *Pharm Res.* 1993;10(2):294-9.

114. **Guirguis MS, Jamali F, Kulmatycki KM, Abouchehade K, Sattari S.** Disease-Drug Interaction: Reduced Response to Propranolol Despite

Increased Concentration in the Rat with Inflammation. *J Pharm Sci.* 2003;92(5):1077-84.

115. **Bishop H, Schneider RE, Welling PG.** Plasma Propranolol Concentrations in Rats with Adjuvant-Induced Arthritis. *Biopharm Drug Dispos.* 1981;2(3):291-7.

116. **Yasuhara M, Fujiwara J, Kitade S, Katayama H, Okumura K, Hori R.** Effect of Altered Plasma Protein Binding on Pharmacokinetics and Pharmacodynamics of Propranolol in Rats after Surgery: Role of Alpha-1-Acid Glycoprotein. *J Pharmacol Exp Ther.* 1985;235(2):513-520.

117. **Laethem ME, Belpaire FM, Wijnant P, Rosseel MT, Bogaert MG.** Influence of Endotoxin on the Stereoselective Pharmacokinetics of Oxprenolol, Propranolol, and Verapamil in the Rat. *Chirality.* 1994;6(5):405-10.

118. **McTavish D, Sorkin EM.** Verapamil. An Updated Review of Its Pharmacodynamic and Pharmacokinetic Properties, and Therapeutic Use in Hypertension. *Drugs.* 1989;38(1):19-76.

119. **Abernethy DR, Wainer IW, Longstreth JA, Andrawis NS.** Stereoselective Verapamil Disposition and Dynamics in Aging During Racemic Verapamil Administration. *J Pharmacol Exp Ther.* 1993;266(2):904-11.

120. **Abernethy DR, Schwartz JB.** Verapamil Pharmacodynamics and Disposition in Obese Hypertensive Patients. *J Cardiovasc Pharmacol.* 1988;11(2):209-15.

121. **Sermsappasuk P, Abdelrahman O, Weiss M.** Cardiac Pharmacokinetics and Inotropic Response of Verapamil in Rats with Endotoxemia. *J Pharm Sci.* 2007.

122. **Sattari S, Dryden WF, Eliot LA, Jamali F.** Despite Increased Plasma Concentration, Inflammation Reduces Potency of Calcium Channel Antagonists Due to Lower Binding to the Rat Heart. *Br J Pharmacol.* 2003;139(5):945-54.

123. **Brocks DR.** Drug Disposition in Three Dimensions: An Update on Stereoselectivity in Pharmacokinetics. *Biopharm Drug Dispos.* 2006;27(8):387-406.

124. **Kulmatycki KM, Abouchehade K, Sattari S, Jamali F.** Drug-Disease Interactions: Reduced Beta-Adrenergic and Potassium Channel Antagonist Activities of Sotalol in the Presence of Acute and Chronic Inflammatory Conditions in the Rat. *Br J Pharmacol.* 2001;133(2):286-94.

125. **Kulmatycki KM, Jamali F.** Pharmacodynamics of Sotolol Enantiomers Are Influenced by Inflammation with No Pharmacokinetic Consequence. *Pharm Res.* 1997;14:S511.

126. **Burnier M.** Angiotensin II Type 1 Receptor Blockers. *Circulation.* 2001;103(6):904-12.

127. **Daneshtalab N, Lewanczuk RZ, Russell AS, Jamali F.** Drug-Disease Interactions: Losartan Effect Is Not Downregulated by Rheumatoid Arthritis. *The Journal of Clinical Pharmacology.* 2006;46(11):1344-1355.

128. **Daneshtalab N, Lewanczuk RZ, Russell A, Jamali F.** Rheumatoid Arthritis Does Not Reduce the Pharmacodynamic Response to Valsartan. *J Clin Pharmacol.* 2004;44(3):245-252.

129. **Brodde O-E, Michel MC.** Adrenergic and Muscarinic Receptors in the Human Heart. *Pharmacol Rev.* 1999;51(4):651-690.

130. **Sarsero D, Russell FD, Lynham JA, Rabnott G, Yang I, Fong KM, Li L, Kaumann AJ, Molenaar P.** (-)-Cgp 12177 Increases Contractile Force and Hastens Relaxation of Human Myocardial Preparations through a Propranolol-Resistant State of the β_1-Adrenoceptor. *Naunyn-Schmiedeberg's Archives of Pharmacology.* 2003;V367(1):10-21.

131. **Granneman JG.** The Putative β_4-Adrenergic Receptor Is a Novel State of the β_1-Adrenergic Receptor. *Am J Physiol Endocrinol Metab.* 2001;280(2):E199-202.

132. **Guimarães S, Moura D.** Vascular Adrenoceptors: An Update. *Pharmacol Rev.* 2001;53(2):319-356.

133. **Wallukat G.** The Beta-Adrenergic Receptors. *Herz.* 2002;27(7):683-90.

134. **Thawornkaiwong A, Preawnim S, Wattanapermpool J.** Upregulation of β_1-Adrenergic Receptors in Ovariectomized Rat Hearts. *Life Sciences.* 2003;72(16):1813-1824.

135. **Freedman NJ, Lefkowitz RJ.** Anti- β_1-Adrenergic Receptor Antibodies and Heart Failure: Causation, Not Just Correlation. *J. Clin. Invest.* 2004;113(10):1379-1382.

136. **Freedman NJ, Liggett SB, Drachman DE, Pei G, Caron MG, Lefkowitz RJ.** Phosphorylation and Desensitization of the Human β_1-Adrenergic Receptor. *J. Biol. Chem.* 1995;270(30):17953-17961.

137. **He J, Xu J, Castleberry AM, Lau AG, Hall RA.** Glycosylation of β_1-Adrenergic Receptors Regulates Receptor Surface Expression and Dimerization. *Biochemical and Biophysical Research Communications.* 2002;297(3):565-572.

138. **Summers RJ, Kompa A, Roberts SJ.** Adrenoceptor Subtypes and Their Desensitization Mechanisms. *J Auto Pharmacol.* 1997;17(6):331-343.

139. **Evanko DS, Ellis CE, Venkatachalam V, Frielle T.** Preliminary Analysis of the Transcriptional Regulation of the Human β_1-Adrenergic Receptor Gene,. *Biochemical and Biophysical Research Communications.* 1998;244(2):395-402.

140. **McDevitt DG.** In Vivo Studies on the Function of Cardiac Beta-Adrenoceptors in Man. *Eur Heart J.* 1989;10(Suppl B):22-8.

141. **Hellgren I, Sylven C, Magnusson Y.** Study of the β_1 Adrenergic Receptor Expression in Human Tissues: Immunological Approach. *Biol Pharm Bull.* 2000;23(6):700-3.

142. **Wang YG, Samarel AM, Lipsius SL.** Laminin Binding to β_1-Integrins Selectively Alters β_1- and β_2-Adrenoceptor Signalling in Cat Atrial Myocytes. *J Physiol (Lond).* 2000;527(1):3-9.

143. **Brouri F, Hanoun N, Mediani O, Saurini F, Hamon M, Vanhoutte PM, Lechat P.** Blockade of [Beta]$_1$- and Desensitization of [Beta]2-

Adrenoceptors Reduce Isoprenaline-Induced Cardiac Fibrosis. *European Journal of Pharmacology*. 2004;485(1-3):227-234.

144. **Ostrom RS, Gregorian C, Drenan RM, Xiang Y, Regan JW, Insel PA.** Receptor Number and Caveolar Co-Localization Determine Receptor Coupling Efficiency to Adenylyl Cyclase. *J. Biol. Chem.* 2001;276(45):42063-42069.

145. **Wang T, Brown MJ.** Differential Expression of Adenylyl Cyclase Subtypes in Human Cardiovascular System. *Molecular and Cellular Endocrinology.* 2004;223(1-2):55-62.

146. **Börjesson M, Magnusson Y, Hjalmarson Å, Andersson B.** A Novel Polymorphism in the Gene Coding for the β_1-Adrenergic Receptor Associated with Survival in Patients with Heart Failure. *European Heart Journal.* 2000;21(22):1853-1858.

147. **Moore JD, Mason DA, Green SA, Hsu J, Liggett SB.** Racial Differences in the Frequencies of Cardiac β_1-Adrenergic Receptor Polymorphisms: Analysis of C145a>G and C1165g>C. *Hum Mutat.* 1999;14(3):271.

148. **Small KM, Wagoner LE, Levin AM, Kardia SLR, Liggett SB.** Synergistic Polymorphisms of β_1- and β_{2c}-Adrenergic Receptors and the Risk of Congestive Heart Failure. *N Engl J Med.* 2002;347(15):1135-1142.

149. **Michel MC, Insel PA.** Receptor Gene Polymorphisms: Lessons on Functional Relevance from the β_1-Adrenoceptor. *Br J Pharmacol.* 2003;138(2):279-282.

150. **ExPASy**. (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (SIB). [Web page] 2007 [cited 2007 January 9]; Swiss-Prot: B1AR_HUMAN (P08588)]. Available from: http://ca.expasy.org/uniprot/P08588.

151. **Rapacciuolo A, Suvarna S, Barki-Harrington L, Luttrell LM, Cong M, Lefkowitz RJ, Rockman HA.** Protein Kinase a and G Protein-Coupled Receptor Kinase Phosphorylation Mediates β_1 Adrenergic Receptor Endocytosis through Different Pathways. *J. Biol. Chem.* 2003;278(37):35403-35411.

152. **Jahns R, Boivin V, Hein L, Triebel S, Angermann CE, Ertl G, Lohse MJ.** Direct Evidence for a β_1-Adrenergic Receptor-Directed Autoimmune Attack as a Cause of Idiopathic Dilated Cardiomyopathy. *J. Clin. Invest.* 2004;113(10):1419-1429.

153. **Iwata M, Yoshikawa T, Baba A, Anzai T, Nakamura I, Wainai Y, Takahashi T, Ogawa S.** Autoimmunity against the Second Extracellular Loop of β_1-Adrenergic Receptors Induces β-Adrenergic Receptor Desensitization and Myocardial Hypertrophy in Vivo. *Circ Res.* 2001;88(6):578-586.

154. **Pierce KL, Premont RT, Lefkowitz RJ.** Seven-Transmembrane Receptors. *Nat Rev Mol Cell Biol.* 2002;3(9):639-650.

155. **Yatani A, Tajima Y, Green SA.** Coupling of β-Adrenergic Receptors to Cardiac L-Type Ca_2^+ Channels: Preferential Coupling of the β_1 Versus β_2

Receptor Subtype and Evidence for PKA-Independent Activation of the Channel. *Cellular Signalling.* 1999;11(5):337-342.

156. **van der Heyden MA, Wijnhoven TJ, Opthof T.** Molecular Aspects of Adrenergic Modulation of Cardiac L-Type Ca_2^+ Channels. *Cardiovasc Res.* 2005;65(1):28-39.

157. **Bootman MD, Higazi DR, Coombes S, Roderick HL.** Calcium Signalling During Excitation-Contraction Coupling in Mammalian Atrial Myocytes. *J Cell Sci.* 2006;119(19):3915-3925.

158. **Layland J, Solaro RJ, Shah AM.** Regulation of Cardiac Contractile Function by Troponin I Phosphorylation. *Cardiovascular Research.* 2005;66(1):12-21.

159. **Okawa Y, Adachi A, Ino M, Nagumo S, Matsui H.** Further Characterization of the 5'-Flanking Promoter Region of the Human $ß_1$-Adrenergic Receptor Gene. *Psychiatry Clin Neurosci.* 1998;52(4):453-458.

160. **Tseng Y-T, Stabila JP, Nguyen TT, McGonnigal BG, Waschek JA, Padbury JF.** A Novel Glucocorticoid Regulatory Unit Mediates the Hormone Responsiveness of the $ß_1$-Adrenergic Receptor Gene. *Molecular and Cellular Endocrinology.* 2001;181(1-2):165-178.

161. **Myslivecek J, Ricny J, Kolar F, Tucek S.** The Effects of Hydrocortisone on Rat Heart Muscarinic and Adrenergic Alpha $_1$, Beta $_1$ and Beta $_2$ Receptors, Propranolol-Resistant Binding Sites and on Some Subsequent Steps in Intracellular Signalling. *Naunyn Schmiedebergs Arch Pharmacol.* 2003;368(5):366-76. Epub 2003 Oct 17.

162. **Thawornkaiwong A, Preawnim S, Wattanapermpool J.** Upregulation of β_1-Adrenergic Receptors in Ovariectomized Rat Hearts. *Life Sciences.* 2003;72(16):1813-1824.

163. **Tseng YT, Stabila J, McGonnigal B, Nguyen TT, Padbury JF.** An Inverted Camp Response Element Mediates the Camp Induction of the Ovine β-Adrenergic Receptor Gene. *Biochem Mol Biol Int.* 1998;46(6):1127-34.

164. **Adli H, Bazin R, Perret GY.** Interaction of Amiodarone and Triiodothyronine on the Expression of β-Adrenoceptors in Brown Adipose Tissue of Rat. *Br J Pharmacol.* 1999;126(6):1455-1461.

165. **Kirigiti P, Bai Y, Yang Y-F, Li X, Li B, Brewer G, Machida CA.** Agonist-Mediated Down-Regulation of Rat β_1-Adrenergic Receptor Transcripts: Role of Potential Post-Transcriptional Degradation Factors. *Mol Pharmacol.* 2001;60(6):1308-1324.

166. **Dunigan CD, Hoang Q, Curran PK, Fishman PH.** Complexity of Agonist- and Cyclic Amp-Mediated Downregulation of the Human β_1-Adrenergic Receptor: Role of Internalization, Degradation, and Mrna Destabilization. *Biochemistry.* 2002;41(25):8019-30.

167. **Ping P, Gelzer-Bell R, Roth DA, Kiel D, Insel PA, Hammond HK.** Reduced Beta-Adrenergic Receptor Activation Decreases G-Protein Expression and Beta-Adrenergic Receptor Kinase Activity in Porcine Heart. *J Clin Invest.* 1995;95(3):1271-80.

168. **Brodde O-E**, Clinical Significance of ß-Adrenergic Receptors, in *Adrenoceptors - Structure, Function and Pharmacology*, RR Ruffolo, Jr., Editor. 1995, Harwood Academic Publisher: Luxembourg. 259-267.

169. **Bernardi M, Deslauriers R, Docherty J, Rossi C, Rossini L, Rossini P, Tonnini C.** Spectral Analysis of Intercycle Heart Fluctuations in the Diethyl-Ether-Anaesthetized or Pithed Rat Treated with Prazosin, DL-Propranolol, Endothelin-1, Alpha-R Atriopeptin and Ace-Inhibitors. *J Auton Pharmacol.* 1998;18(5):271-80.

170. **Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC, Cohen RJ.** Power Spectrum Analysis of Heart Rate Fluctuation: A Quantitative Probe of Beat-to-Beat Cardiovascular Control. *Science.* 1981;213(4504):220-2.

171. **Blaxall BC, Pellett AC, Wu SC, Pende A, Port JD.** Purification and Characterization of β-Adrenergic Receptor Mrna-Binding Proteins. 2000;275(6):4290-4297.

172. **Pende A, Tremmel KD, DeMaria CT, Blaxall BC, Minobe WA, Sherman JA, Bisognano JD, Bristow MR, Brewer G, Port JD.** Regulation of the Mrna-Binding Protein AUF1 by Activation of the Beta-Adrenergic Receptor Signal Transduction Pathway. *J Biol Chem.* 1996;271(14):8493-8501.

173. **Iaccarino G, Tomhave ED, Lefkowitz RJ, Koch WJ.** Reciprocal in Vivo Regulation of Myocardial G Protein–Coupled Receptor Kinase Expression by ß-Adrenergic Receptor Stimulation and Blockade. *Circulation.* 1998;98(17):1783-1789.

174. **Koch WJ, Rockman HA, Samama P, Hamilton RA, Bond RA, Milano CA, Lefkowitz RJ.** Cardiac Function in Mice Overexpressing the Beta-Adrenergic Receptor Kinase or a Beta Ark Inhibitor. *Science.* 1995;268(5215):1350-3.

175. **Tse J, Huang MW, Leone RJ, Weiss HR, He YQ, Scholz PM.** Down Regulation of Myocardial Beta$_1$-Adrenoceptor Signal Transduction System in Pacing-Induced Failure in Dogs with Aortic Stenosis-Induced Left Ventricular Hypertrophy. *Mol Cell Biochem.* 2000;205(1-2):67-73.

176. **Anthonio RL, Brodde O-E, van Veldhuisen DJ, Scholtens E, Crijns HJGM, van Gilst WH.** B-Adrenoceptor Density in Chronic Infarcted Myocardium: A Subtype Specific Decrease of β_1-Adrenoceptor Density. *International Journal of Cardiology.* 2000;72(2):137-141.

177. **Gulick T, Chung MK, Pieper SJ, Lange LG, Schreiner GF.** Interleukin 1 and Tumor Necrosis Factor Inhibit Cardiac Myocyte β -Adrenergic Responsiveness. *PNAS.* 1989;86(17):6753-6757.

178. **Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R.** Peroxynitrite Is a Major Contributor to Cytokine-Induced Myocardial Contractile Failure. *Circ Res.* 2000;87(3):241-247.

179. **Boillot A, Massol J, Maupoil V, Grelier R, Capellier G, Berthelot A, Barale F.** Alterations of Myocardial and Vascular Adrenergic Receptor-Mediated Responses in Escherichia Coli-Induced Septic Shock in the Rat. *Crit Care Med.* 1996;24(8):1373-80.

180. **Krown KA, Yasui K, Brooker MJ, Dubin AE, Nguyen C, Harris GL, McDonough PM, Glembotski CC, Palade PT, Sabbadini RA.** Tnf[Alpha] Receptor Expression in Rat Cardiac Myocytes: TNFα Inhibition of L-Type Ca_2^+ Current and Ca_2^+ Transients. *FEBS Letters.* 1995;376(1-2):24-30.

181. **Longhurst JC, Tjen-A-Looi SC, Fu L-W.** Cardiac Sympathetic Afferent Activation Provoked by Myocardial Ischemia and Reperfusion: Mechanisms and Reflexes. *Ann NY Acad Sci.* 2001;940(1):74-95.

182. **Brodde O-E, Leineweber K.** Autonomic Receptor Systems in the Failing and Aging Human Heart: Similarities and Differences. *European Journal of Pharmacology.* 2004;500(1-3):167-176.

183. **Nian M, Lee P, Khaper N, Liu P.** Inflammatory Cytokines and Postmyocardial Infarction Remodeling. *Circ Res.* 2004;94(12):1543-1553.

184. **Fairweather D, Rose NR.** Inflammatory Heart Disease: A Role for Cytokines. *Lupus.* 2005;14(9):646-51.

185. **Härle P, Straub RH, Wiest R, Mayer A, Scholmerich J, Atzeni F, Carrabba M, Cutolo M, Sarzi-Puttini P.** Increase of Sympathetic Outflow Measured by Neuropeptide Y and Decrease of the Hypothalamic-Pituitary-Adrenal Axis Tone in Patients with Systemic Lupus Erythematosus and Rheumatoid Arthritis: Another Example of Uncoupling of Response Systems. *Ann Rheum Dis.* 2006;65(1):51-56.

186. **Tanaka H, Ueta Y, Yamashita U, Kannan H, Yamashita H.** Biphasic Changes in Behavioral, Endocrine, and Sympathetic Systems in Adjuvant Arthritis in Lewis Rats. *Brain Research Bulletin.* 1996;39(1):33-37.

187. **Rathz DA, Brown KM, Kramer LA, Liggett SB.** Amino Acid 49 Polymorphisms of the Human Beta1-Adrenergic Receptor Affect Agonist-Promoted Trafficking. *J Cardiovasc Pharmacol.* 2002;39(2):155-60.

188. **Levin MC, Marullo S, Muntaner O, Andersson B, Magnusson Y.** The Myocardium-Protective Gly-49 Variant of the Beta $_1$-Adrenergic Receptor Exhibits Constitutive Activity and Increased Desensitization and Down-Regulation. *J. Biol. Chem.* 2002;277(34):30429-30435.

189. **Mason DA, Moore JD, Green SA, Liggett SB.** A Gain-of-Function Polymorphism in a G-Protein Coupling Domain of the Human Beta $_1$-Adrenergic Receptor. *J. Biol. Chem.* 1999;274(18):12670-12674.

190. **Ryden M, Hoffstedt J, Eriksson P, Bringman S, Arner P.** The Arg 389 Gly Beta1-Adrenergic Receptor Gene Polymorphism and Human Fat Cell Lipolysis. *Int J Obes Relat Metab Disord.* 2001;25(11):1599-603.

191. **Ellsworth DL, Coady SA, Chen W, Srinivasan SR, Boerwinkle E, Berenson GS.** Interactive Effects between Polymorphisms in the β-Adrenergic Receptors and Longitudinal Changes in Obesity. *Obes Res.* 2005;13(3):519-526.

192. **Tesson F, Charron P, Peuchmaurd M, Nicaud V, Cambien F, Tiret L, Poirier O, Desnos M, Jullieres Y, Amouyel P.** Characterization of a Unique Genetic Variant in the β_1-Adrenoceptor Gene and Evaluation of Its Role in Idiopathic Dilated Cardiomyopathy. *Journal of Molecular and Cellular Cardiology.* 1999;31(5):1025-1032.

193. **Brodde OE, Bruck H, Leineweber K.** Cardiac Adrenoceptors: Physiological and Pathophysiological Relevance. *J Pharmacol Sci.* 2006;100(5):323-37.

194. **Liggett SB, Mialet-Perez J, Thaneemit-Chen S, Weber SA, Greene SM, Hodne D, Nelson B, Morrison J, Domanski MJ, Wagoner LE, Abraham WT, Anderson JL, Carlquist JF, et al.** A Polymorphism within a Conserved Beta$_1$-Adrenergic Receptor Motif Alters Cardiac Function and Beta-Blocker Response in Human Heart Failure. *Proc Natl Acad Sci USA.* 2006;103(30):11288-11293.

195. **Wahle M, Krause A, Pierer M, Hantzschel H, Baerwald CGO.** Immunopathogenesis of Rheumatic Diseases in the Context of Neuroendocrine Interactions. *Ann NY Acad Sci.* 2002;966(1):355-364.

196. **Straub RH, Cutolo M.** Involvement of the Hypothalamic--Pituitary--Adrenal/Gonadal Axis and the Peripheral Nervous System in Rheumatoid Arthritis: Viewpoint Based on a Systemic Pathogenetic Role. *Arthritis Rheum.* 2001;44(3):493-507.

197. **Elenkov IJ, Chrousos GP.** Stress Hormones, Proinflammatory and Antiinflammatory Cytokines, and Autoimmunity. *Ann N Y Acad Sci.* 2002;966:290-303.

198. **Harbuz MS, Rees RG, Lightman SL.** HPA Axis Responses to Acute Stress and Adrenalectomy During Adjuvant-Induced Arthritis in the Rat. *Am J Physiol Regul Integr Comp Physiol.* 1993;264(1):R179-185.

199. **Straub R, Dhabhar F, Bijlsma J, Cutolo M.** How Psychological Stress Via Hormones and Nerve Fibers May Exacerbate Rheumatoid Arthritis. *Arthritis & Rheumatism.* 2005;52(1):16-26.

200. **Sarlis N, Chowdrey H, Stephanou A, Lightman S.** Chronic Activation of the Hypothalamo-Pituitary-Adrenal Axis and Loss of Circadian Rhythm During Adjuvant-Induced Arthritis in the Rat. *Endocrinology.* 1992;130(4):1775-1779.

201. **Kin NW, Sanders VM.** It Takes Nerve to Tell T and B Cells What to Do. *J Leukoc Biol.* 2006;79(6):1093-1104.

202. **Haskó G.** Receptor-Mediated Interaction between the Sympathetic Nervous System and Immune System in Inflammation. *Neurochemical Research.* 2001;26(8 - 9):1039-1044.

203. **Straub RH, Dhabhar FS, Bijlsma JW, Cutolo M.** How Psychological Stress Via Hormones and Nerve Fibers May Exacerbate Rheumatoid Arthritis. *Arthritis Rheum.* 2005;52(1):16-26.

204. **Miller LE, Justen H-P, Scholmerich J, Straub RH.** The Loss of Sympathetic Nerve Fibers in the Synovial Tissue of Patients with Rheumatoid Arthritis Is Accompanied by Increased Norepinephrine Release from Synovial Macrophages. *FASEB J.* 2000;14(13):2097-2107.

205. **Backs J, Haunstetter A, Gerber SH, Metz J, Borst MM, Strasser RH, Kubler W, Haass M.** The Neuronal Norepinephrine Transporter in Experimental Heart Failure: Evidence for a Posttranscriptional Downregulation. *J Mol Cell Cardiol.* 2001;33(3):461-72.

206. **Mandela P, Ordway GA.** The Norepinephrine Transporter and Its Regulation. *Journal of Neurochemistry.* 2006;97(2):310-333.

207. **Ungerer M, Chlistalla A, Richardt G.** Upregulation of Cardiac Uptake1 Carrier in Ischemic and Nonischemic Rat Heart. *Circ Res.* 1996;78(6):1037-1043.

208. **Esler M, Jennings G, Lambert G, Meredith I, Horne M, Eisenhofer G.** Overflow of Catecholamine Neurotransmitters to the Circulation: Source, Fate, and Functions. *Physiol. Rev.* 1990;70(4):963-985.

209. **Kopin IJ, Rundqvist B, Friberg P, Lenders J, Goldstein DS, Eisenhofer G.** Different Relationships of Spillover to Release of Norepinephrine in Human Heart, Kidneys, and Forearm. *Am J Physiol Regul Integr Comp Physiol.* 1998;275(1):R165-173.

210. **Hasking G, Esler M, Jennings G, Burton D, Johns J, Korner P.** Norepinephrine Spillover to Plasma in Patients with Congestive Heart Failure: Evidence of Increased Overall and Cardiorenal Sympathetic Nervous Activity. *Circulation.* 1986;73(4):615-621.

211. **Jayanthi LD, Annamalai B, Samuvel DJ, Gether U, Ramamoorthy S.** Phosphorylation of the Norepinephrine Transporter at Threonine 258 and Serine 259 Is Linked to Protein Kinase C-Mediated Transporter Internalization. *J Biol Chem.* 2006;281(33):23326-23340.

212. **Haughey HM, Brown JM, Fleckenstein AE, Hanson GR.** Oxygen Radicals Differentially Affect Na^+/Cl^--Dependent Transporters. *European Journal of Pharmacology.* 1999;379(1):107-110.

213. **Delehanty JM, Himura Y, Elam H, Hood WB, Jr, Liang CS.** Beta-Adrenoceptor Downregulation in Pacing-Induced Heart Failure Is Associated with Increased Interstitial NE Content. *Am J Physiol Heart Circ Physiol.* 1994;266(3):H930-935.

214. **Borst MM, Beuthien W, Schwencke C, LaRosee P, Marquetant R, Haass M, Kubler W, Strasser RH.** Desensitization of the Pulmonary Adenylyl Cyclase System : A Cause of Airway Hyperresponsiveness in Congestive Heart Failure? *Journal of the American College of Cardiology.* 1999;34(3):848-856.

215. **Ungerer M, Weig H-J, Kubert S, Overbeck M, Bengel F, Schomig A, Schwaiger M.** Regional Pre- and Postsynaptic Sympathetic System in the Failing Human Heart – Regulation of βARK-1. *European Journal of Heart Failure.* 2000;2(1):23-31.

216. **Reithmann C, Gierschik P, Jakobs KH, Werdan K.** Regulation of Adenylyl Cyclase by Noradrenaline and Tumour Necrosis Factor Alpha in Rat Cardiomyocytes. *Eur Heart J.* 1991;12(Suppl F):139-42.

217. **Cutolo M, Sulli A, Pizzorni C, Craviotto C, Straub RH.** Hypothalamic-Pituitary-Adrenocortical and Gonadal Functions in Rheumatoid Arthritis. *Ann NY Acad Sci.* 2003;992(1):107-117.

218. **Kuis W, de Jong-de Vos van Steenwijk CCE, Sinnema G, Kavelaars A, Prakken B, Helders PJM, Heijnen CJ.** The Autonomic Nervous System and the Immune System in Juvenile Rheumatoid Arthritis. *Brain, Behavior, and Immunity.* 1996;10(4):387-398.

219. **Gozke E, Erdogan N, Akyuz G, Turan B, Akyuz E, Us O.** Sympathetic Skin Response and R-R Interval Variation in Cases with Rheumatoid Arthritis. *Electromyogr Clin Neurophysiol.* 2003;43(2):81-4.

220. **Perry F, Heller PH, Kamiya J, Levine JD.** Altered Autonomic Function in Patients with Arthritis or with Chronic Myofascial Pain. *Pain.* 1989;39(1):77-84.

221. **Pogwizd SM, Bers DM.** Cellular Basis of Triggered Arrhythmias in Heart Failure. *Trends in Cardiovascular Medicine.* 2004;14(2):61-66.

222. **MERIT-HF_Study_Group.** Effect of Metoprolol CR/XL in Chronic Heart Failure: Metoprolol CR/XL Randomised Intervention Trial in-Congestive Heart Failure (MERIT-HF). *The Lancet.* 1999;353(9169):2001-2007.

223. **Loftus EV, Jr.** A Matter of Life or Death: Mortality in Crohn's Disease. *Inflamm Bowel Dis.* 2002;8(6):428-9.

224. **Mouzas IA, Pallis AG, Kochiadakis GE, Marketou M, Chlouverakis GI, Mellisas J, Vardas PE, Kouroumalis EA.** Autonomic Imbalance During the Day in Patients with Inflammatory Bowel Disease in Remission. Evidence from Spectral Analysis of Heart Rate Variability over 24 Hours. *Digestive and Liver Disease.* 2002;34(11):775-780.

225. **Kanik KS, Chrousos GP, Schumacher HR, Crane ML, Yarboro CH, Wilder RL.** Adrenocorticotropin, Glucocorticoid, and Androgen Secretion in Patients with New Onset Synovitis/Rheumatoid Arthritis: Relations with Indices of Inflammation. *J Clin Endocrinol Metab.* 2000;85(4):1461-1466.

226. **Szekanecz Z, Halloran MM, Volin MV, Woods JM, Strieter RM, Kenneth Haines G, 3rd, Kunkel SL, Burdick MD, Koch AE.** Temporal Expression of Inflammatory Cytokines and Chemokines in Rat Adjuvant-Induced Arthritis. *Arthritis Rheum.* 2000;43(6):1266-77.

227. **Kader HA, Tchernev VT, Satyaraj E, Lejnine S, Kotler G, Kingsmore SF, Patel DD.** Protein Microarray Analysis of Disease Activity in Pediatric Inflammatory Bowel Disease Demonstrates Elevated Serum Plgf, IL-7, Tgf- β_1, and IL-12p40 Levels in Crohn's Disease and Ulcerative Colitis Patients in Remission Versus Active Disease. *Am J Gastroenterology.* 2005;100(2):414-423.

228. **Grip O, Janciauskiene S, Lindgren S.** Circulating Monocytes and Plasma Inflammatory Biomarkers in Active Crohn's Disease: Elevated Oxidized Low-Density Lipoprotein and the Anti-Inflammatory Effect of Atorvastatin. *Inflamm Bowel Dis.* 2004;10(3):193-200.

229. **Gustot T, Lemmers A, Louis E, Nicaise C, Quertinmont E, Belaiche J, Roland S, Van Gossum A, Deviere J, Franchimont D.** Profile of Soluble Cytokine Receptors in Crohn's Disease. *Gut.* 2005;54(4):488-495.

230. **Oudkerk Pool M, Bouma G, Visser JJ, Kolkman JJ, Tran DD, Meuwissen SG, Pena AS.** Serum Nitrate Levels in Ulcerative Colitis and Crohn's Disease. *Scand J Gastroenterol.* 1995;30(8):784-8.

231. **Singh K, Communal C, Sawyer DB, Colucci WS.** Adrenergic Regulation of Myocardial Apoptosis. *Cardiovascular Research.* 2000;45(3):713-719.

232. **Blum A, Miller H.** Pathophysiological Role of Cytokines in Congestive Heart Failure. *Annu Rev Med.* 2001;52:15-27.

233. **Watson AM, Hood SG, May CN.** Mechanisms of Sympathetic Activation in Heart Failure. *Clin Exp Pharmacol Physiol.* 2006;33(12):1269-74.

234. **Engelhardt S, Hein L, Dyachenkow V, Kranias EG, Isenberg G, Lohse MJ.** Altered Calcium Handling Is Critically Involved in the Cardiotoxic Effects of Chronic β-Adrenergic Stimulation. *Circulation.* 2004;109(9):1154-1160.

235. **Kaye DM, Lefkovits J, Jennings GL, Bergin P, Broughton A, Esler MD.** Adverse Consequences of High Sympathetic Nervous Activity in the Failing Human Heart. *Journal of the American College of Cardiology.* 1995;26(5):1257-1263.

236. **Reiken S, Gaburjakova M, Gaburjakova J, He K-I, Prieto A, Becker E, Yi G-h, Wang J, Burkhoff D, Marks AR.** β-Adrenergic Receptor Blockers Restore Cardiac Calcium Release Channel (Ryanodine Receptor) Structure and Function in Heart Failure. *Circulation.* 2001;104(23):2843-2848.

237. **Bristow MR.** β-Adrenergic Receptor Blockade in Chronic Heart Failure. *Circulation.* 2000;101(5):558-569.

238. **Munch G, Rosport K, Bultmann A, Baumgartner C, Li Z, Laacke L, Ungerer M.** Cardiac Overexpression of the Norepinephrine Transporter Uptake-1 Results in Marked Improvement of Heart Failure. *Circ Res.* 2005;97(9):928-936.

239. **Kawai H, Mohan A, Hagen J, Dong E, Armstrong J, Stevens SY, Liang CS.** Alterations in Cardiac Adrenergic Terminal Function and Beta-Adrenoceptor Density in Pacing-Induced Heart Failure. *Am J Physiol Heart Circ Physiol.* 2000;278(5):H1708-16.

240. **National Institute for Clinical Excellence UK**, *Guidance on the Use of Cyclo-Oxygenase (COX) II Selective Inhibitors, Celecoxib, Rofecoxib, Meloxicam and Etodolac for Osteoarthritis and Rheumatoid Arthritis.* 2001, London.

241. **Piquette-Miller M, Jamali F.** Influence of Severity of Inflammation on the Disposition Kinetics of Propranolol Enantiomers in Ketoprofen-Treated and Untreated Adjuvant Arthritis. *Drug Metab Dispos.* 1995;23(2):240-5.

242. **Chakravarty K, McDonald H, Pullar T, Taggart A, Chalmers R, Oliver S, Mooney J, Somerville M, Bosworth A.** BSR & BHPR Guideline for Disease-Modifying Anti-Rheumatic Drug (DMARD) Therapy in Consultation with the British Association of Dermatologists. *Rheumatology (Oxford).* 2006.

243. **Toyohira Y, Yanagihara N, Minami K, Ueno S, Uezono Y, Tachikawa E, Kondo Y, Kashimoto T, Izumi F.** Down-Regulation of the Noradrenaline Transporter by Interferon-Alpha in Cultured Bovine Adrenal Medullary Cells. 1998;70(4):1441-1447.

244. **Li W, Knowlton D, Woodward WR, Habecker BA.** Regulation of Noradrenergic Function by Inflammatory Cytokines and Depolarization. *J Neurochem.* 2003;86(3):774-83.

245. **Buss SJ, Backs J, Kreusser MM, Hardt SE, Maser-Gluth C, Katus HA, Haass M.** Spironolactone Preserves Cardiac Norepinephrine Reuptake in Salt-Sensitive Dahl Rats. *Endocrinology*. 2006;147(5):2526-2534.

246. **National Institute for Clinical Excellence UK**, *Adalimumab, Etanercept and Infliximab for the Treatment of Rheumatoid Arthritis*. 2007, London.

247. **Kayo S, Katsuya N, Yoshinori K, Kazuhiko Y**. Angiotensin Receptor Blockers Suppress Antigen-Specific T Cell Responses and Ameliorate Collagen-Induced Arthritis in Mice. 2005;52(6):1920-1928.

248. *Statins: The HMG-CoA Reductase Inhibitors in Perspective*. 2nd ed, ed. A Gaw, CJ Packard, and J Shepherd. 2004, London: Martin Dunitz.

249. **Lennernas H, Fager G.** Pharmacodynamics and Pharmacokinetics of the HMG-CoA Reductase Inhibitors. Similarities and Differences. *Clin Pharmacokinet.* 1997;32(5):403-25.

250. **California Environmental Protection Agency**. Office of Environmental Health Hazard Assessment. [Web Page] 2003 [cited 2007 December 6].

251. **Shepherd J, Cobbe SM, Ford IAN, Isles CG, Lorimer AR, Macfarlane PW, McKillop JH, Packard CJ.** Prevention of Coronary Heart Disease with Pravastatin in Men with Hypercholesterolemia. *Atherosclerosis Supplements*. 2004;5(3):91-97.

252. West of Scotland Coronary Prevention Study: Implications for Clinical Practice. The WOSCOPS Study Group. *Eur Heart J.* 1996;17(2):163-4.

253. **Sacks FM, Pfeffer MA, Moye LA, Rouleau JL, Rutherford JD, Cole TG, Brown L, Warnica JW, Arnold JM, Wun CC, Davis BR, Braunwald E.**

The Effect of Pravastatin on Coronary Events after Myocardial Infarction in Patients with Average Cholesterol Levels. Cholesterol and Recurrent Events Trial Investigators. *N Engl J Med.* 1996;335(14):1001-9.

254. Prevention of Cardiovascular Events and Death with Pravastatin in Patients with Coronary Heart Disease and a Broad Range of Initial Cholesterol Levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. *N Engl J Med.* 1998;339(19):1349-57.

255. **Takemoto M, Liao JK.** Pleiotropic Effects of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase Inhibitors. *Arterioscler Thromb Vasc Biol.* 2001;21(11):1712-9.

256. **LaRosa JC, He J, Vupputuri S.** Effect of Statins on Risk of Coronary Disease: A Meta-Analysis of Randomized Controlled Trials. *JAMA.* 1999;282(24):2340-2346.

257. **McFarlane SI, Muniyappa R, Francisco R, Sowers JR.** Clinical Review 145: Pleiotropic Effects of Statins: Lipid Reduction and Beyond. *J Clin Endocrinol Metab.* 2002;87(4):1451-8.

258. **Franzoni F, Quinones-Galvan A, Regoli F, Ferrannini E, Galetta F.** A Comparative Study of the in Vitro Antioxidant Activity of Statins. *Int J Cardiol.* 2003;90(2-3):317-21.

259. **Cohen LH, Pieterman E, van Leeuwen REW, Overhand M, Burm BEA, van der Marel GA, van Boom JH.** Inhibitors of Prenylation of RAS and

Other G-Proteins and Their Application as Therapeutics. *Biochemical Pharmacology*. 2000;60(8):1061-1068.

260. **Takeuchi S, Kawashima S, Rikitake Y, Ueyama T, Inoue N, Hirata K-i, Yokoyama M.** Cerivastatin Suppresses Lipopolysaccharide-Induced ICAM-1 Expression through Inhibition of Rho GTPase in BAEC. *Biochemical and Biophysical Research Communications*. 2000;269(1):97-102.

261. **Etienne-Manneville S, Hall A.** Rho GTPases in Cell Biology. *Nature*. 2002;420(6916):629-35.

262. **Martin G, Duez H, Blanquart C, Berezowski V, Poulain P, Fruchart J-C, Najib-Fruchart J, Glineur C, Staels B.** Statin-Induced Inhibition of the Rho-Signaling Pathway Activates PPAR-alpha and Induces HDL ApoA-I. *J. Clin. Invest.* 2001;107(11):1423-1432.

263. **Bourcier T, Libby P.** Hmg Coa Reductase Inhibitors Reduce Plasminogen Activator Inhibitor-1 Expression by Human Vascular Smooth Muscle and Endothelial Cells. *Arterioscler Thromb Vasc Biol*. 2000;20(2):556-62.

264. **Albert MA, Danielson E, Rifai N, Ridker PM.** Effect of Statin Therapy on C-Reactive Protein Levels: The Pravastatin Inflammation/CRP Evaluation (Prince): A Randomized Trial and Cohort Study. *JAMA*. 2001;286(1):64-70.

265. **Arnaud C, Burger F, Steffens S, Veillard NR, Nguyen TH, Trono D, Mach F.** Statins Reduce Interleukin-6-Induced C-Reactive Protein in

Human Hepatocytes: New Evidence for Direct Antiinflammatory Effects of Statins. *Arterioscler Thromb Vasc Biol.* 2005;25(6):1231-6.

266. **Takahashi HK, Iwagaki H, Yagi T, Kanke T, Liu K, Yoshino T, Tanaka N, Nishibori M.** Action Profiles of Statins and Calcineurin Inhibitors During Human Mixed Lymphocyte Reaction. *Clin Immunol.* 2007;123(3):324-32.

267. **Yamagata T, Kinoshita K, Nozaki Y, Sugiyama M, Ikoma S, Funauchi M.** Effects of Pravastatin in Murine Collagen-Induced Arthritis. *Rheumatol Int.* 2007;27(7):631-9.

268. **Inoue I, Goto S-i, Mizotani K, Awata T, Mastunaga T, Kawai S-i, Nakajima T, Hokari S, Komoda T, Katayama S.** Lipophilic Hmg-Coa Reductase Inhibitor Has an Anti-Inflammatory Effect: Reduction of Mrna Levels for Interleukin-1β, Interleukin-6, Cyclooxygenase-2, and P22phox by Regulation of Peroxisome Proliferator-Activated Receptor [Alpha] (PPAR[Alpha]) in Primary Endothelial Cells. *Life Sciences.* 2000;67(8):863-876.

269. **Li TS, Takahashi M, Suzuki R, Kobayashi T, Ito H, Mikamo A, Hamano K.** Pravastatin Improves Remodeling and Cardiac Function after Myocardial Infarction by an Antiinflammatory Mechanism Rather Than by the Induction of Angiogenesis. *Ann Thorac Surg.* 2006;81(6):2217-25.

270. **Rosenson RS, Tangney CC, Casey LC.** Inhibition of Proinflammatory Cytokine Production by Pravastatin. *The Lancet.* 1999;353(9157):983-984.

271. **Zelvyte I, Dominaitiene R, Crisby M, Janciauskiene S.** Modulation of Inflammatory Mediators and PPAR[Gamma]and Nf[Kappa]B Expression

by Pravastatin in Response to Lipoproteins in Human Monocytes in Vitro. *Pharmacological Research.* 2002;45(2):147-154.

272. **Crisby M, Nordin-Fredriksson G, Shah PK, Yano J, Zhu J, Nilsson J.** Pravastatin Treatment Increases Collagen Content and Decreases Lipid Content, Inflammation, Metalloproteinases, and Cell Death in Human Carotid Plaques: Implications for Plaque Stabilization. *Circulation.* 2001;103(7):926-33.

273. **Grip O, Janciauskiene S, Lindgren S.** Pravastatin Down-Regulates Inflammatory Mediators in Human Monocytes in Vitro. *Eur J Pharmacol.* 2000;410(1):83-92.

274. **Porreca E, Di Febbo C, Baccante G, Di Nisio M, Cuccurullo F.** Increased Transforming Growth Factor-Beta1 Circulating Levels and Production in Human Monocytes after 3-Hydroxy-3-Methyl-Glutaryl-Coenzyme a Reductase Inhibition with Pravastatin. *Journal of the American College of Cardiology.* 2002;39(11):1752-1757.

275. **Hori A, Kanda Y, Goyama S, Onishi Y, Komeno Y, Mitani K, Kishi Y, Ogawa S, Imataki O, Chiba S, Kojima R, Hamaki T, Sakiyama M, et al.** A Prospective Trial to Evaluate the Safety and Efficacy of Pravastatin for the Treatment of Refractory Chronic Graft-Versus-Host Disease. *Transplantation.* 2005;79(3):372-4.

276. **Rudich SM, Mongini PKA, Perez RV, Katznelson S.** Hmg-Coa Reductase Inhibitors Pravastatin and Simvastatin Inhibit Human B-Lymphocyte Activation. *Transplantation Proceedings.* 1998;30(4):992-995.

277. **Sukhova GK, Williams JK, Libby P.** Statins Reduce Inflammation in Atheroma of Nonhuman Primates Independent of Effects on Serum Cholesterol. *Arterioscler Thromb Vasc Biol.* 2002;22(9):1452-8.

278. **Katznelson S, Wang XM, Chia D, Ozawa M, Zhong HP, Hirata M, Terasaki PI, Kobashigawa JA.** The Inhibitory Effects of Pravastatin on Natural Killer Cell Activity in Vivo and on Cytotoxic T Lymphocyte Activity in Vitro. *J Heart Lung Transplant.* 1998;17(4):335-40.

279. **Hilgendorff A, Muth H, Parviz B, Staubitz A, Haberbosch W, Tillmanns H, Holschermann H.** Statins Differ in Their Ability to Block NF-Kappa-B Activation in Human Blood Monocytes. *Int J Clin Pharmacol Ther.* 2003;41(9):397-401.

280. **Forman BM, Chen J, Evans RM.** Hypolipidemic Drugs, Polyunsaturated Fatty Acids, and Eicosanoids Are Ligands for Peroxisome Proliferator-Activated Receptors Alpha and Delta. *PNAS.* 1997;94(9):4312-4317.

281. **Willson TM, Brown PJ, Sternbach DD, Henke BR.** The PPARs: From Orphan Receptors to Drug Discovery. *J Med Chem.* 2000;43(4):527-50.

282. **Francis GA, Annicotte JS, Auwerx J.** PPAR Agonists in the Treatment of Atherosclerosis. *Curr Opin Pharmacol.* 2003;3(2):186-91.

283. **Francis GA, Fayard E, Picard F, Auwerx J.** Nuclear Receptors and the Control of Metabolism. *Annual Review of Physiology.* 2003;65(1):261-311.

284. **Senftleben U, Karin M.** The IKK/NF-Kappa B Pathway. *Crit Care Med.* 2002;30(1 Suppl):S18-26.

285. **Hilliard B, Samoilova EB, Liu TS, Rostami A, Chen Y.** Experimental Autoimmune Encephalomyelitis in NF-Kappa B-Deficient Mice:Roles of NF-Kappa B in the Activation and Differentiation of Autoreactive T Cells. *J Immunol.* 1999;163(5):2937-43.

286. **Kim I, Moon S-O, Hoon Kim S, Jin Kim H, Soon Koh Y, Young Koh G.** Vascular Endothelial Growth Factor Expression of Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), and E-Selectin through Nuclear Factor-Kappa B Activation in Endothelial Cells. *J. Biol. Chem.* 2001;276(10):7614-7620.

287. **Nath N, Giri S, Prasad R, Singh AK, Singh I.** Potential Targets of 3-Hydroxy-3-Methylglutaryl Coenzyme a Reductase Inhibitor for Multiple Sclerosis Therapy. *J Immunol.* 2004;172(2):1273-1286.

288. **Li Q, Verma IM.** NF-Kappab Regulation in the Immune System. *Nat Rev Immunol.* 2002;2(10):725-34.

289. **Ridker PM, Rifai N, Pfeffer MA, Sacks FM, Moye LA, Goldman S, Flaker GC, Braunwald E.** Inflammation, Pravastatin, and the Risk of Coronary Events after Myocardial Infarction in Patients with Average Cholesterol Levels. Cholesterol and Recurrent Events (Care) Investigators. *Circulation.* 1998;98(9):839-44.

290. **Stewart RAH, White HD, Kirby AC, Heritier SR, Simes RJ, Nestel PJ, West MJ, Colquhoun DM, Tonkin AM, for the Long-Term Intervention With Pravastatin in Ischemic Disease Study Investigators.** White

Blood Cell Count Predicts Reduction in Coronary Heart Disease Mortality with Pravastatin. *Circulation.* 2005;111(14):1756-1762.

291. **Blake GJ, Ridker PM.** Are Statins Anti-Inflammatory? *Curr Control Trials Cardiovasc Med.* 2000;1(3):161-165.

292. **Hamaad A, Sosin M, Lip GY, MacFadyen RJ.** Short-Term Adjuvant Atorvastatin Improves Frequency Domain Indices of Heart Rate Variability in Stable Systolic Heart Failure. *Cardiovasc Drugs Ther.* 2005;19(3):183-7.

293. **Leung BP, Sattar N, Crilly A, Prach M, McCarey DW, Payne H, Madhok R, Campbell C, Gracie JA, Liew FY, McInnes IB.** A Novel Anti-Inflammatory Role for Simvastatin in Inflammatory Arthritis *J Immunol.* 2003;170(3):1524-1530.

294. **Palmer G, Chobaz V, Talabot-Ayer D, Taylor S, So A, Gabay C, Busso N.** Assessment of the Efficacy of Different Statins in Murine Collagen-Induced Arthritis. *Arthritis Rheum.* 2004;50(12):4051-9.

295. **Kanda H, Hamasaki K, Kubo K, Tateishi S, Yonezumi A, Kanda Y, Yamamoto K, Mimura T.** Antiinflammatory Effect of Simvastatin in Patients with Rheumatoid Arthritis. *J Rheumatol.* 2002;29(9):2024-6.

296. **McCarey DW, McInnes IB, Madhok R, Hampson R, Scherbakov O, Ford I, Capell HA, Sattar N.** Trial of Atorvastatin in Rheumatoid Arthritis (TARA): Double-Blind, Randomised Placebo-Controlled Trial. *Lancet.* 2004;363(9426):2015-21.

297. **Tikiz C, Utuk O, Pirildar T, Bayturan O, Bayindir P, Taneli F, Tikiz H, Tuzun C.** Effects of Angiotensin-Converting Enzyme Inhibition and Statin Treatment on Inflammatory Markers and Endothelial Functions in Patients with Longterm Rheumatoid Arthritis. *J Rheumatol.* 2005;32(11):2095-101.

298. **Hermann F, Forster A, Chenevard R, Enseleit F, Hurlimann D, Corti R, Spieker LE, Frey D, Hermann M, Riesen W, Neidhart M, Michel BA, Hellermann JP, et al.** Simvastatin Improves Endothelial Function in Patients with Rheumatoid Arthritis. *Journal of the American College of Cardiology.* 2005;45(3):461-464.

299. **Pliquett RU, Cornish KG, Peuler JD, Zucker IH.** Simvastatin Normalizes Autonomic Neural Control in Experimental Heart Failure. *Circulation.* 2003;107(19):2493-2498.

300. **Davignon J, Mabile L.** [Mechanisms of Action of Statins and Their Pleiotropic Effects]. *Ann Endocrinol (Paris).* 2001;62(1 Pt 2):101-12.

301. **Egashira K, Hirooka Y, Kai H, Sugimachi M, Suzuki S, Inou T, Takeshita A.** Reduction in Serum Cholesterol with Pravastatin Improves Endothelium-Dependent Coronary Vasomotion in Patients with Hypercholesterolemia. *Circulation.* 1994;89(6):2519-24.

302. **Borghi C, Prandin MG, Costa FV, Bacchelli S, Degli Esposti D, Ambrosioni E.** Use of Statins and Blood Pressure Control in Treated Hypertensive Patients with Hypercholesterolemia. *J Cardiovasc Pharmacol.* 2000;35(4):549-55.

303. **Horwich TB, MacLellan WR, Fonarow GC.** Statin Therapy Is Associated with Improved Survival in Ischemic and Non-Ischemic Heart Failure. *J Am Coll Cardiol.* 2004;43(4):642-8.

304. **Jialal I, Stein D, Balis D, Grundy SM, Adams-Huet B, Devaraj S.** Effect of Hydroxymethyl Glutaryl Coenzyme a Reductase Inhibitor Therapy on High Sensitive C-Reactive Protein Levels. *Circulation.* 2001;103(15):1933-5.

305. **Kobashigawa JA, Katznelson S, Laks H, Johnson JA, Yeatman L, Wang XM, Chia D, Terasaki PI, Sabad A, Cogert GA, Trosian K, Hamilton MA, Moriguchi JD, et al.** Effect of Pravastatin on Outcomes after Cardiac Transplantation. *N Engl J Med.* 1995;333(10):621-627.

306. **Lee TM, Chou TF, Tsai CH.** Association of Pravastatin and Left Ventricular Mass in Hypercholesterolemic Patients: Role of 8-Iso-Prostaglandin F_2alpha Formation. *J Cardiovasc Pharmacol.* 2002;40(6):868-74.

307. **Reid IR, Hague W, Emberson J, Baker J, Tonkin A, Hunt D, MacMahon S, Sharpe N.** Effect of Pravastatin on Frequency of Fracture in the Lipid Study: Secondary Analysis of a Randomised Controlled Trial. Long-Term Intervention with Pravastatin in Ischaemic Disease. *Lancet.* 2001;357(9255):509-12.

308. **Nishikawa H, Miura S, Zhang B, Shimomura H, Arai H, Tsuchiya Y, Saku K.** Pravastatin Promotes Coronary Collateral Circulation in Patients with Coronary Artery Disease. *Coron Artery Dis.* 2002;13(7):377-81.

309. **Atthobari J, Brantsma AH, Gansevoort RT, Visser ST, Asselbergs FW, van Gilst WH, de Jong PE, de Jong-van den Berg LT.** The Effect of Statins on Urinary Albumin Excretion and Glomerular Filtration Rate: Results from Both a Randomized Clinical Trial and an Observational Cohort Study. *Nephrol Dial Transplant.* 2006;21(11):3106-14.

310. **ten Dam VH, Box FM, de Craen AJ, van den Heuvel DM, Bollen EL, Murray HM, van Buchem MA, Westendorp RG, Blauw GJ.** Lack of Effect of Pravastatin on Cerebral Blood Flow or Parenchymal Volume Loss in Elderly at Risk for Vascular Disease. *Stroke.* 2005;36(8):1633-6.

311. **Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G.** Decreased Prevalence of Alzheimer Disease Associated with 3-Hydroxy-3-Methyglutaryl Coenzyme A Reductase Inhibitors. *Arch Neurol.* 2000;57(10):1439-43.

312. **Freeman DJ, Norrie J, Sattar N, Neely RD, Cobbe SM, Ford I, Isles C, Lorimer AR, Macfarlane PW, McKillop JH, Packard CJ, Shepherd J, Gaw A.** Pravastatin and the Development of Diabetes Mellitus: Evidence for a Protective Treatment Effect in the West of Scotland Coronary Prevention Study. *Circulation.* 2001;103(3):357-62.

313. **US National Institutes of Health**. Clinical Trials.Gov. 2007 [cited 2007 Dec 8]; Clinical Trials Registry]. Available from: http://clinicaltrials.gov/.

314. **Grisham MB, Johnson GG, Lancaster JR, Jr.** Quantitation of Nitrate and Nitrite in Extracellular Fluids. *Methods Enzymol.* 1996;268:237-46.

315. **Liuzzo G, Biasucci LM, Gallimore JR, Grillo RL, Rebuzzi AG, Pepys MB, Maseri A.** The Prognostic Value of C-Reactive Protein and Serum Amyloid A Protein in Severe Unstable Angina. *N Engl J Med.* 1994;331(7):417-24.

316. **Buono C, Binder CJ, Stavrakis G, Witztum JL, Glimcher LH, Lichtman AH.** T-Bet Deficiency Reduces Atherosclerosis and Alters Plaque Antigen-Specific Immune Responses. *PNAS.* 2005;102(5):1596-1601.

317. **Youssef S, Stuve O, Patarroyo JC, Ruiz PJ, Radosevich JL, Hur EM, Bravo M, Mitchell DJ, Sobel RA, Steinman L, Zamvil SS.** The Hmg-Coa Reductase Inhibitor, Atorvastatin, Promotes a Th2 Bias and Reverses Paralysis in Central Nervous System Autoimmune Disease. *Nature.* 2002;420(6911):78-84.

318. **Paintlia AS, Paintlia MK, Singh AK, Stanislaus R, Gilg AG, Barbosa E, Singh I.** Regulation of Gene Expression Associated with Acute Experimental Autoimmune Encephalomyelitis by Lovastatin. *J Neurosci Res.* 2004;77(1):63-81.

319. **Barsante MM, Roffe E, Yokoro CM, Tafuri WL, Souza DG, Pinho V, Castro MSDA, Teixeira MM.** Anti-Inflammatory and Analgesic Effects of Atorvastatin in a Rat Model of Adjuvant-Induced Arthritis. *European Journal of Pharmacology.* 2005;516(3):282-289.

320. **Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH.** A Novel Transcription Factor, T-Bet, Directs Th1 Lineage Commitment. *Cell.* 2000;100(6):655-69.

321. **Keystone E, Wherry J, Grint P.** IL-10 as a Therapeutic Strategy in the Treatment of Rheumatoid Arthritis. *Rheum Dis Clin North Am.* 1998;24(3):629-39.

322. **Scott DL.** Prognostic Factors in Early Rheumatoid Arthritis. *Rheumatology.* 2000;39(90001):24-29.

323. **de Beer FC, Baltz ML, Munn EA, Feinstein A, Taylor J, Bruton C, Clamp JR, Pepys MB.** Isolation and Characterization of C-Reactive Protein and Serum Amyloid P Component in the Rat. *Immunology.* 1982;45(1):55-70.

324. **Rifai N, Tracy RP, Ridker PM.** Clinical Efficacy of an Automated High-Sensitivity C-Reactive Protein Assay. *Clin Chem.* 1999;45(12):2136-2141.

325. **Goodson N.** Coronary Artery Disease and Rheumatoid Arthritis. *Curr Opin Rheumatol.* 2002;14(2):115-20.

326. **Elward K, Griffiths M, Mizuno M, Harris CL, Neal JW, Morgan BP, Gasque P.** Cd46 Plays a Key Role in Tailoring Innate Immune Recognition of Apoptotic and Necrotic Cells. *J. Biol. Chem.* 2005;280(43):36342-36354.

327. **Sattari S, Dryden W, Jamali F**. Abstract: Inflammation Decreases the Binding of β_1-Adrenergic Receptors in Rat Myocardium. in *17th annual Meeting of American Association of Pharmaceutical Scientists.* 2002. Toronto, Ontario, Canada.

328. **Liu SJ, Zhou W, Kennedy RH.** Suppression of Beta -Adrenergic Responsiveness of L-Type Ca_2+ Current by Il-1β in Rat Ventricular Myocytes. *Am J Physiol Heart Circ Physiol.* 1999;276(1):H141-148.

329. **Lee DS, Austin PC, Rouleau JL, Liu PP, Naimark D, Tu JV.** Predicting Mortality among Patients Hospitalized for Heart Failure: Derivation and Validation of a Clinical Model. *JAMA.* 2003;290(19):2581-2587.

330. **Chindavijak B, Belpaire FM, Bogaert MG.** Effect of Inflammation on the Metabolism of Antipyrine, Lidocaine and Propranolol in Isolated Rat Hepatocytes. *Pharmacology.* 1988;36:279-82.

331. **Fradette C, Du Souich P.** Effect of Hypoxia on Cytochrome P450 Activity and Expression. *Curr Drug Metab.* 2004;5(3):257-71.

332. **Khatsenko O, Gross S, Rifkind A, Vane J.** Nitric Oxide Is a Mediator of the Decrease in Cytochrome P450-Dependent Metabolism Caused by Immunostimulants. *PNAS.* 1993;90(23):11147-11151.

333. **Wagner AH, Schwabe O, Hecker M.** Atorvastatin Inhibition of Cytokine-Inducible Nitric Oxide Synthase Expression in Native Endothelial Cells in Situ. *Br J Pharmacol.* 2002;136(1):143-149.

334. **Griselli M, Herbert J, Hutchinson WL, Taylor KM, Sohail M, Krausz T, Pepys MB.** C-Reactive Protein and Complement Are Important Mediators of Tissue Damage in Acute Myocardial Infarction. *J. Exp. Med.* 1999;190(12):1733-1740.

335. **Bodi I, Mikala G, Koch SE, Akhter SA, Schwartz A.** The L-Type Calcium Channel in the Heart: The Beat Goes On. *J. Clin. Invest.* 2005;115(12):3306-3317.

336. **Kleiner D. E., Stetlerstevenson W. G.** Quantitative Zymography: Detection of Picogram Quantities of Gelatinases. *Analytical Biochemistry.* 1994;218(2):325-329.

337. **Schulz R**, Intracellular Targets of Matrix Metalloproteinase-2 in Cardiac Disease: Rationale and Therapeutic Approaches. 2007. 211-242.

338. **Feldmann M, Brennan FM, Maini RN.** Role of Cytokines in Rheumatoid Arthritis. *Annual Review of Immunology.* 1996;14(1):397-440.

339. **Mayer AF, Schroeder C, Heusser K, Tank J, Diedrich A, Schmieder RE, Luft FC, Jordan J.** Influences of Norepinephrine Transporter Function on the Distribution of Sympathetic Activity in Humans. *Hypertension.* 2006;48(1):120-126.

340. **Gao L, Wang W, Li Y-L, Schultz HD, Liu D, Cornish KG, Zucker IH.** Superoxide Mediates Sympathoexcitation in Heart Failure: Roles of Angiotensin II and NAD(P)H Oxidase. *Circulation Research.* 2004;95(9):937-944.

341. **Gao L, Wang W, Li YL, Schultz HD, Liu D, Cornish KG, Zucker IH.** Simvastatin Therapy Normalizes Sympathetic Neural Control in Experimental Heart Failure: Roles of Angiotensin II Type 1 Receptors and NAD(P)H Oxidase. *Circulation.* 2005;112(12):1763-70.

342. **Kishi T, Hirooka Y, Mukai Y, Shimokawa H, Takeshita A.** Atorvastatin Causes Depressor and Sympatho-Inhibitory Effects with Upregulation of Nitric Oxide Synthases in Stroke-Prone Spontaneously Hypertensive Rats. *J Hypertens.* 2003;21(2):379-86.

343. **Selmeczy Z, Szelenyi J, Vizi ES.** Intact Noradrenaline Transporter Is Needed for the Sympathetic Fine-Tuning of Cytokine Balance. *European Journal of Pharmacology.* 2003;469(1-3):175-181.

344. **Qun Gao C, Sawicki G, Suarez-Pinzon WL, Csont T, Wozniak M, Ferdinandy P, Schulz R.** Matrix Metalloproteinase-2 Mediates Cytokine-Induced Myocardial Contractile Dysfunction. *Cardiovascular Research.* 2003;57(2):426-433.

345. **Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JRB, Sawicki G, Schulz R.** Intracellular Action of Matrix Metalloproteinase-2 Accounts for Acute Myocardial Ischemia and Reperfusion Injury. *Circulation.* 2002;106(12):1543-1549.

346. **Gerhardstein BL, Gao T, Bunemann M, Puri TS, Adair A, Ma H, Hosey MM.** Proteolytic Processing of the C Terminus of the Alpha(1c) Subunit of L-Type Calcium Channels and the Role of a Proline-Rich Domain in Membrane Tethering of Proteolytic Fragments. *J Biol Chem.* 2000;275(12):8556-63.

347. **Kamp TJ, Hell JW.** Regulation of Cardiac L-Type Calcium Channels by Protein Kinase A and Protein Kinase C. *Circ Res.* 2000;87(12):1095-1102.

348. **Ganesan AN, Maack C, Johns DC, Sidor A, O'Rourke B.** {Beta}-Adrenergic Stimulation of L-Type Ca2+ Channels in Cardiac Myocytes Requires the Distal Carboxyl Terminus of {Alpha}1c but Not Serine 1928. *Circ Res.* 2006;98(2):e11-18.

349. **Gao T, Cuadra AE, Ma H, Bunemann M, Gerhardstein BL, Cheng T, Eick RT, Hosey MM.** C-Terminal Fragments of the Alpha 1c (Cav1.2) Subunit Associate with and Regulate L-Type Calcium Channels Containing C-Terminal-Truncated Alpha 1c Subunits. *The Journal of Biological Chemistry.* 2001;276(24):21089-21097.

350. **Woo S-H, Soldatov NM, Morad M.** Modulation of Ca2+ Signalling in Rat Atrial Myocytes: Possible Role of the Alpha1c Carboxyl Terminal. *J Physiol.* 2003;552(2):437-447.

351. **Chen J, Zhang XM, Xu Q.** Involvement of Lymphocytes with a Th1 Cytokine Profile in Bone Cell Damage Associated with MMP-9 Production in Collagen-Induced Arthritis. *Inflammation Research.* 2004;53(12):670-679.

352. **Lombardi MS, Kavelaars A, Schedlowski M, Bijlsma JWJ, Okihara KL, Van De Pol M, Ochsmann S, Pawlak C, Schmidt RE, Heijnen CJ.** Decreased Expression and Activity of G-Protein-Coupled Receptor Kinases in Peripheral Blood Mononuclear Cells of Patients with Rheumatoid Arthritis. *FASEB J.* 1999;13(6):715-725.

353. **Kubota T, McNamara DM, Wang JJ, Trost M, McTiernan CF, Mann DL, Feldman AM.** Effects of Tumor Necrosis Factor Gene Polymorphisms on

Patients with Congestive Heart Failure. *Circulation.* 1998;97(25):2499-2501.

354. **Paoletti R, Gotto AM, Jr, Hajjar DP.** Inflammation in Atherosclerosis and Implications for Therapy. *Circulation.* 2004;109(23_suppl_1):III-20-26.

355. **McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY.** A Novel Anti-Inflammatory Role of Simvastatin in a Murine Model of Allergic Asthma. *J Immunol.* 2004;172(5):2903-2908.

356. **Thongtang V, Srivanasont N, Chaithiraphan S.** Hemodynamic Effects of a Beta Adrenergic Blocking Agent-Atenolol in Patients with Severe Mitral Stenosis. *J Med Assoc Thai.* 1993;76(1):9-16.

357. **Carson RP, Appalsamy M, Diedrich A, Davis TL, Robertson D.** Animal Model of Neuropathic Tachycardia Syndrome. *Hypertension.* 2001;37(6):1357-1361.

358. **Perlini S, Ferrero I, Palladini G, Tozzi R, Gatti C, Vezzoli M, Cesana F, Janetti MB, Clari F, Busca G, Mancia G, Ferrari AU.** Survival Benefits of Different Antiadrenergic Interventions in Pressure Overload Left Ventricular Hypertrophy/Failure. *Hypertension.* 2006;48(1):93-97.

359. **Kompa AR, Molenaar P, Summers RJ.** Effect of Chemical Sympathectomy on (-)-Isoprenaline-Induced Changes in Cardiac Beta-Adrenoceptor Subtypes in the Guinea-Pig and Rat. *J Auton Pharmacol.* 1994;14(6):411-23.

360. **Marano G, Ramirez A, Mori I, Ferrari AU.** Sympathectomy Inhibits the Vasoactive Effects of Nicotine in Conscious Rats. *Cardiovascular Research.* 1999;42(1):201-205.

361. **Ferrari A, Daffonchio A, Franzelli C, Mancia G.** Potentiation of the Baroreceptor-Heart Rate Reflex by Sympathectomy in Conscious Rats. *Hypertension.* 1991;18(2):230-235.

362. **Kim LR, Whelpdale K, Zurowski M, Pomeranz B.** Sympathetic Denervation Impairs Epidermal Healing in Cutaneous Wounds. *Wound Repair and Regeneration.* 1998;6(3):194-201.

363. **Hansson M, Kjorell U, Forsgren S.** Increased Immunoexpression of Atrial Natriuretic Peptide in the Heart Conduction System of the Rat after Cardiac Sympathectomy. *Journal of Molecular and Cellular Cardiology.* 1998;30(10):2047-2057.

364. **Mircoli L, Fedele L, Benetti M, Bolla GB, Radaelli A, Perlini S, Ferrari AU.** Preservation of the Baroreceptor Heart Rate Reflex by Chemical Sympathectomy in Experimental Heart Failure. *Circulation.* 2002;106(7):866-872.

365. **Sterin-Borda L, Leiros CP, Borda ES, de Bracco MME.** Effects of Il-2 on the Myocardium. Participation of the Sympathetic System. *Journal of Molecular and Cellular Cardiology.* 1996;28(12):2457-2465.